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19. ABSTRACT (Continue on reverse if necessary and identify by block number) We have shown that immunoaberrant trauma patients' MØ produce abnormally elevated levels of IL-6, PGE_2, TGF_β, as well as TNF_α. These patients' MØ TNF_α are insensitive to PGE_2 downregulation and largely consist of cell-associated TNF_α. The *in vivo* activated MØ from these trauma patients are aberrant in their response to the cyclo-oxygenase inhibitor indomethacin in that their TNF_α levels are abnormally augmented by Indo. These patients' abnormal increase in MØ TNF_α levels in response to Indo appear to be a result of increased sensitivity to stimulation by lipoxygenase products, particularly LTB_4. Crosslinking the patients' MØ $Fc_\gamma RI$, as would occur *in vivo* by excessive circulating immunoglobulin, results in exaggerated production of TNF_α, IL-6 and PGE_2 by patients' MØ. We showed that immunoaberrant patients' MØ also have increased sensitivity to TGF_β, and that excessive MØ produced TGF_β is available in the post-trauma microenvironment. The post-trauma activated MØ produces both greater PGE_2 activity and augmented levels of cell-associated TNF_α in response to the activated TGF_β in its microenvironment. The PGE_2 and TNF_α responses of normal MØ that have been preactivated by $Fc_\gamma RI$ crosslinking and then exposed to TGF_β parallels the patients' MØ responses in kind but not quantity.

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We have examined indomethacin, lypoxygenase inhibitors, synthetic glucans and interleukin-4, as possible immunomodulators post-trauma. Indo, as described above, was effective in PGE_2 downregulation, but massively increased MØ cell-asscciated TNF_α levels. The addition of a lypoxygenase inhibitor prevented this TNF_α augmentation, but had no effect on post-trauma MØ PGE_2 levels, indicating a possible combinational therapy of Indo and a lypoxygenase inhibitor. Synthetic glucans would decrease post-trauma MØ PGE_2 levels and TNF_α levels, but had no effect on MØ IL-6 production, indicating they might be useful in post-trauma prophylactic therapy. IL-4 was effective in downregulating patients' elevated MØ PGE_2 activity, but was less effective in correcting the patients' MØ aberrant TNF_α responses. Altered MØ response potentials can be predicted by assessing the trauma patients' MØ <u>in vitro</u> for their response to potential mediators or immunotherapeutic modalities.

During this contract period we have made substantial progress in characterizing those dysfunctions which are most critical in the post-trauma septic syndrome. In addition, we have defined the interactions of these aberrant MØ functions. We have adapted a number of new assays for specifically assessing patients' MØ functions. We have also explored a number of possible prophylactic therapies.

During this period, we have shown that immunoaberrant trauma patients' MØ produce abnormally elevated levels of IL-6, PGE_2, TGF_β, as well as TNF_α. These patients' MØ TNF_α are insensitive to PGE_2 downregulation and largely consist of cell-associated TNF_α. The in vivo activated MØ from these trauma patients are aberrant in their response to the cyclo-oxygenase inhibitor indomethacin in that their TNF_α levels are abnormally augmented by Indo. These patients' abnormal increase in MØ TNF_α levels in response to Indo appear to be a result of increased sensitivity to stimulation by lipoxygenase products, particularly LTB_4. The patients' trauma activated MØ are not just more sensitive to LTB_4 but also to other trauma induced mediators in the microenvironment. Crosslinking the patients' MØ $Fc_\gamma RI$, as would occur in vivo by excessive circulating immunoglobulin, results in exaggerated production of TNF_α, IL-6 and PGE_2 by patients' MØ. We have also shown that immunoaberrant patients' MØ also have increased sensitivity to TGF_β. Excessive MØ produced TGF_β is available in the post-trauma microenvironment (14). This TGF_β is activated from its latent form by MØ produced proteolytic enzymes (49). The post-trauma MØ is now autocrine stimulated by its own TGF_β. The post-trauma activated MØ produces both greater PGE_2 activity and augmented levels of cell-associated TNF_α in response to the activated TGF_β in its microenvironment. The normal unactivated MØ is unaffected or even depressed by exogenous TGF_β. The PGE_2 and TNF_α responses of normal MØ that have been preactivated by $Fc_\gamma RI$ crosslinking and then exposed to TGF_β parallels the patients' MØ responses in kind but not quantity.

The response of trauma-activated patients' cells is very different from normals' and leads to questions about the actual response of patients' MØ to in vivo therapy. We have examined indomethacin, lypoxygenase inhibitors, synthetic glucans and interleukin-4, as possible immunomodulators post-trauma. Indo, as described above, was effective in PGE_2 downregulation, but massively increased MØ cell-associated TNF_α levels. The addition of a lypoxygenase inhibitor prevented this TNF_α augmentation, but had no effect on post-trauma MØ PGE_2 levels. Consequently, a combinational therapy of Indo and a lypoxygenase inhibitor may be indicated. Synthetic glucans would decrease post-trauma MØ PGE_2 levels and TNF_α levels, but had no effect on MØ IL-6 production. This inexpensive drug might have broad usage in post-trauma prophylactic therapy. IL-4 was effective in downregulating patients' elevated MØ PGE_2 activity, but was less effective in correcting the patients' MØ aberrant TNF_α responses. The unusual activation of trauma patients' MØ by trauma-generated mediators like complement split products, circulating immunoglobulin and fibrin degradation products etc., results in a preactivated monocyte. The subsequent response of this in vivo activated MØ to bacterial stimuli or other trauma-generated mediators varies greatly from that of normals' monocytes. These altered MØ response potentials can be predicted by assessing the trauma patients' MØ in vitro for their response to potential mediators or immunotherapeutic modalities.

This contract has met all the goals set for it and expanded the information available on the treatment and pathology of trauma patients. This data is directly applicable in the care of combat casualties and should aid in the Army's mission of the best care and early return to duty for its members.

FOREWORD

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

The investigators have abided by the "National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules", April 1982, and Administrative Practices supplements.

Carol L. Miller Gregware
PI Signature Date

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Introduction to Problem under Study:

This contract, when proposed six and a half years ago, had three separate but related foci. First, we were to develop new assays to monitor those post-trauma monocyte functions which contribute to the development of immune suppression and sepsis in the trauma and burn patients. Second, we were to define those cellular regulatory dysfunctions which were augmented post-injury and which were pivotal in the development of the post-trauma septic syndrome. Finally, we were proposing to examining different immunomodulators as possible therapeutic treatments to ameliorate the defined immune dysfunctions in the burn and trauma patients.

Although our goals have remained the same over the six year period since the contract was written, there has been a dramatic shift in our experimental protocols and assay systems, reflecting the dramatic advances in understanding of cytokine activities and their role in septic shock syndrome. For example, we originally proposed to measure leukocyte pyrogen levels from patients' and normals' MØ by measuring changes in the rectal temperatures of mice. Our data showed that leukocyte pyrogen was increased in the trauma patients who developed septic episodes. We now measure the individual cytokines (IL-6, TNF_α, and IL-1) which together mediate leukocyte pyrogen activity.

In goal two, we initially focused on experiments to measure suppressor monocyte (MØ) functions. We now measure the production and effect of specific immunoregulatory monokines, including Transforming Growth Factor β, (TGF_β) Prostaglandin E_2 (PGE_2), Tumor Necrosis Factor α (TNF_α) and are initiating investigation of IL-10. Experiments in the third area of concentration examined possible immunomodulatory therapy. We have re-focused our attention on cytokine and inducers which will specifically ameliorate the trauma altered cytokine production rather than examining molecules like thymopoietin, which have a nonspecific, broad ranged, immunostimulatory effect. In summary, this contract has produced reports which more clearly elucidate the altered responses to infection induced by severe injury.

Background:

The septic shock syndrome remains a major cause of morbidity and mortality in trauma and burn patients. Abnormal macrophage production of inflammatory mediators (both cytokines and other products) are proposed to play a pivotal role in the post-trauma septic shock syndrome (6-15). This laboratory originally proposed a key role for the monocyte/macrophage (MØ) in the septic shock syndrome seen after trauma (16). That critical role of the MØ in both burn and trauma immunoaberrations is now generally recognized (17-19).

Characterization of MØ function and mediator actions after severe injury is complicated by the interactive nature of the MØ products and the unusual activation status of the post-trauma MØ. During this contract, we have shown that the response of pre-septic trauma patients' MØ to bacterial stimuli is exaggerated when compared to normals' (20,21). One of our major findings, which have been confirmed by others, is that MØ from trauma patients not only produce elevated tumor necrosis factor α (TNF_α) levels, but that the TNF_α which patients' MØ produce is primarily cell-associated, rather than secreted TNF_α (20,22,23). We have also demonstrated that the trauma patients' MØ are often resistant to PGE_2 downregulation (15,22). Our hypothesis explaining this post-trauma aberrant monokine function is that the microenvironment around trauma patients' MØ consists of high concentrations of secondary inducers of monokines. Examples of these secondary inducers include substance P, immunoglobulin monomers, and complement split products, as well as elevated cytokines (20,24). It is the concomitant presence of these trauma-induced mediators during subsequent MØ

induction that results in deranged monocyte responses, such as exaggerated production of some monokines and depression of both MØ antigen presenting function and plasminogen activator production. During this five year contract, we have defined a number of altered MØ response capacities of trauma patients which appear to correlate with immune aberrations. In addition, we have adapted a number of assays to monitor these MØ dysfunctions. Finally, we have examined a number of possible prophylactic therapies for their specific effect on the dysfunctions we have defined.

Methods:

Separation of monocytes: MØ are separated from patients' and normals' peripheral blood by Ficoll-Hypaque density centrifugation and selective adherence to microexudate treated plates as previously described. MØ purity by this separation technique is 95-97% as determined by FITC-labeled monoclonal MY9 antibody staining. Since lymphocytes can also produce many of the same cytokines we are assessing, MØ purity is essential. $Fc_\gamma RI$ MØ subsets are separated by rosetting the T cell depleted MØ with anti-Rh coated erythrocytes. The rosetting MØ represent the $Fc_\gamma RI$ positive MØ subpopulation, which bears the type I Fc-gamma receptors in high density. The non-rosetting MØ are the $Fc_\gamma RI$ negative MØ subpopulation and express no or very low densities of $Fc_\gamma RI$. $Fc_\gamma RI^{+\gamma}$ and $Fc_\gamma RI^{-}$ MØ subpopulations are greater than 95% positive with the MØ specific OKM5 fluorescenated antibody staining. MØ viability is >97% by trypan blue staining. Limited MØ samples results in not all experiments being run on all patient samples.

Monocyte Stimulation: MØ are isolated from each patient as early as 1-3 days post-injury, and blood is collected biweekly during their hospitalization. Each patient's blood sample is processed along with a normal's control blood donated by the research and hospital staff at the UMMC. MØ were stimulated with 20 μg/ml muramyl dipeptide (MDP) alone, with a combination of 10^{-6} M indomethacin (Indo) + MDP or with a suboptimal dose of interferon gamma (IFN_γ) (10U/ml) plus 20 μg/ml MDP. In some experiments MØ were primed for four hours with 0.05-50 ng/ml IL-4 followed by 20 μg/ml MDP stimulation. The optimal concentration of IL-4 was 5 ng/ml, which has been used in the subsequent assays.

In certain experiments, particulate and/or soluble glucans were employed for MØ stimulation. Glucan B and R4 were used in a concentration range of 10-50 μg/ml and 20 μg/ml was the optimal dose. Soluble glucans RA and RB were used at 20μg/ml. Particulate glucan D served for structural glucan control, without biological effect. MØ supernates were collected after 16-20 hours incubation and the adherent MØ were collected with short EDTA treatment followed by scraping. MØ were used for cell-associated TNF_α and PCA determinations after freezing-thawing and sonication.

Plasminogen Activator (PA) assay, Lysozyme and Procoagulant Activity Measurements: The Plasminogen Activator (PA) assay is performed as previously described: (1) The procoagulant assay and the lysozyme assay are also performed as previously described (25-26).

TNF_α determination: Secreted MØ TNF_α in the MØ supernates and cell-associated MØ TNF_α in the MØ lysates is measured utilizing the L-M cell bioassay as we have published (22). Both cell-associated and secreted MØ TNF_α activity was totally abolished by anti-TNF_α neutralizing antibody. A TNF_α ELISA is also occasionally utilized for confirmation.

IL-6 bioassay: The B9 and MH60/BSF2 murine hybridoma cell lines are maintained in RMPI medium supplemented with 10% FBS and hybridoma growth factor (IL-6). $2x10^4$ B9 cells/well are cultured with serial dilutions of MØ supernates in a 96 well plate as we have published (13). B9 proliferation is measured by ^{3}H-thymidine incorporation during

the last 18 hours of the 72 hour proliferation assay. IL-6 activity of each [illegible] supernatant sample is calculated from the recombinant IL-6 standard based on the following formula: sample act = st. act x $2^{\text{sample titer} - \text{st. titer}}$. An IL-6 [illegible] has also been occasionally used.

Bioassay for TGF_β determination: Mv1Lu, mink lung cells, are maintained in MEM medium containing 10% FBS. 2.5×10^4 Mv1Lu cells/well are cultured with serial dilutions of acid-treated MØ supernates for 26 hours. Inhibition of Mv1Lu cell proliferation by TGF_β is measured by ^{3}H-TDR incorporation during the last 6 hours of the assay. MØ TGF_β activity is calculated from the dilution of the MØ sample and TGF_β standard resulting in half maximal proliferation by the formula described for IL-6 (14).

PGE_2 determination: MØ PGE_2 production is measured in the MØ supernates utilizing the highly sensitive ELISA method as we have published (22).

MIP-1 and IL-8 production: An IL-8 sensitive ELISA was obtained from Sandoz, Vienna, Austria. Besides the highly specific ELISA method, bioactivity of MIP-1 will be measured based on its ability to activate respiratory burst in polymorphonuclear leukocytes (PMNL). MIP-1 induced elevations in PMNL oxidative metabolisms is evaluated using the flow cytometric assay of Zeller (27).

RNA extraction and hybridizations: MØ were harvested by EDTA treatment as previously described (14). Cells were washed with PBS and resuspended in 10mM Vanadyl-Ribonucleoside Complex (Sigma). Total cellular RNA was extracted using the Acid Guanidinium Thiocyanate-Phenol-Chloroform method described by Chomczynski and Sacchi (28). Northern blots were prepared as described after fractionation of RNA on a 1% agarose gel containing 0.66M formaldehyde, then transferred to Nytron membrane by capillary technique. The membranes were baked at 80°C for 1 hour then hybridized with a ^{32}P-labeled cytokine cDNA probe. The human IL-1B probe used in these experiments was a 1.3kb Pst I cDNA fragment. The human IL-6 probe was a 1.2kb EcoRI cDNA fragment. These probes were generously provided by Dr. S. Clark (Genetics Institute, Cambridge, MA). The cDNA restriction fragments were radiolabeled by the random primer method of Feinberg and Fogelstein (29) to a specific activity of 10^8 cpm/mg. Hybridizations were performed with 10^6 cpm of denatured probe/ml hybridization buffer. Prehybridization, hybridization and washing for the Northern blots were performed according to the manufacturer's specifications. The blots were visualized by autoradiography.

Epics Analysis: For determination of the percentage of MØ with different surface markers in the adherence separated MØ of post-trauma patients and normal controls, we increasingly utilized our newly obtained EPICS FACS Analyzer. Direct fluorescent staining with FITC-labeled monoclonal antibodies is performed in our assays. FITC-labeled, matching type of mouse IgG control is always included for the determination of non-specific binding. Briefly, 1×10^6 MØ are stained with the appropriate test-amounts of FITC-labeled MY9, MY4, CD8, CD3, $Fc_\gamma RI$ or $Fc_\gamma RII$ monoclonal antibodies, respectively. After 30 minutes incubation at 4°C, cells are washed three times to remove the excess antibody and resuspended in 1.0 ml resuspension media. Then, the cells are analyzed for fluorescence intensity by the previously established analysis program on the EPICS. The percentage of positive MØ for each surface marker are compared between normals' and patients' MØ.

In experiments studying the TNF_α receptor expression, matrix adherent MØ and $Fc_\gamma RI$ crosslinked MØ ($FC_\gamma RI^+$) were further stimulated in some experiments with 20μg/ml muramyl dipeptide (MDP) or with combinations of a 2 hr. prime with $100U/10^6$ MØ/ml IFN_γ plus an additional 1 hr. of 20μg/ml MDP or with $100U/10^6$ MØ/ml IFN_γ alone. Some MØ were continuously stimulated over an 18 hr. period after their initial 3 hr. prime period. Each MØ stimuli combination was assessed for $TNF_\alpha R$ expression by labeling 10^5 cells in 0.035ml with TNF_α - phycoerythrin (0.7μg/ml) for 60'. MØ with buffer were used as controls. Fluorescent measurements were done on the Coulter EPICS profile with a

530 SP (green) and 590 LP (red) emission filter.

Initially, double labeling experiments were performed with both TNF$_\alpha$-phycoerythrin and the MØ specific monoclonal MY9-FITC to allow gating of MØ. Double labeled samples were measured, using appropriate color compensation, and control cells reacted with an isotype control SgG26-FITC. Gates were generated from histograms of forward light scatter vs MY9 labeling and then by light histograms in further TNF$_\alpha$R experiments. Percent TNF$_\alpha$R positive cells were derived after adjusting the measurement window so that >1% of control cells were positive. TNF$_\alpha$R determinations are reported both as % TNF$_\alpha$R positive cells and as mean TNF$_\alpha$R fluorescent intensity of 10^4 measured MØ. The LM bioassay was used for TNF$_\alpha$ determinations at 3, 8, and 18 hrs. of incubation, measuring secreted TNF$_\alpha$ in the MØ supernates or cell-associated TNF$_\alpha$ in the sonicated MØ lysates after repeated freeze-thawing.

Statistical data analysis: Statistical differences in the unstimulated and stimulated monokine levels between post-trauma patients and normals will be compared using Wilcoxon signed rank or Mann-Whitney U non-parametric analysis. Significant interindividual differences at the level of monokine responses occur in humans. For example, HLA-Class II DR2- and DQw1-positive normal subjects are associated with low TNF$_\alpha$ production, whereas DR3- and DR4-positive individuals almost always show high levels of TNF$_\alpha$ production. Consequently, presentation of our data as mean ± standard error would not be appropriate. Data is presented as the median with range and statistically analyzed with non parametic techniques such as the Wilcoxon or Mann-Whitney U tests.

Results and Discussion:

MØ Aberrations: During contract years one and two, we assessed post-trauma MØ function by assessing the plasminogen activator production, PGE_2 levels, and leukocyte pyrogen activity as indicators of altered MØ action. During this time, we utilized an RIA for measurement of PGE_2. Supernates containing MØ products were collected from patient or control MØ and the PGE_2 activity and leukocyte pyrogen activity assessed. The MØ themselves were used to assess PA production, using the ^{125}I-fibrinogen plates. Since our culture media contained fetal bovine sera, assessment of PGE_2 and LP in the supernates was complicated. The RIA results were often not as quantitative as we desired. In addition, the base line of the LP assays (i.e. murine basal temperature) was often elevated because of endotoxin in the sera obscuring some of the elevation in LP activity. Nevertheless, we were able to demonstrate that patient MØ LP synthesis and PGE_2 production were massively elevated in the trauma and burn patient while plasminogen activator production was decreased (Table 1). These data also showed that LP (i.e. cytokine) activity was increased concomitant to PGE_2 elevation. Our overall assessment of MØ PGE_2 values were low because of the insensitivity of the RIA, but the relative relationship between patients' and normals' MØ values was the same as we presently see with the 10x more sensitive PGE_2 ELISA we presently utilize. The observed elevation in some patients' MØ functions, while others were comcomitantly depressed, lead us to question whether different patients' MØ subsets might be responding differently after severe trauma.

Papers at that time suggested that subsets of MØ might be regulatory or facilitory for immune function, depending on their expression of the Fc$_\gamma$RI receptor for immunoglobulin G (30,31). Using this rossetting method, we separated the patients' and normals' MØ into high density Fc$_\gamma$RI (Fc$_\gamma$RI$^+$) and low density Fc$_\gamma$RI (Fc$_\gamma$RI$^-$) cells. One of our exciting findings at that time was that the immunosuppressed patients showed an increased percentage of MØ which had high densities of the Fc$_\gamma$RI receptor (Fc$_\gamma$RI$^+$MØ) (Fig.1). We also showed that the increase in the proportion of these Fc$_\gamma$RI$^+$ MØ occurred over time post-trauma (Fig.2). We were also able to show that the Fc$_\gamma$RI MØ both in normals and in patients produced the majority of their PGE_2 activity (Fig.3).

Finally, we showed that the MØ population, which was decreased in these patients, (i.e. low density $Fc_\gamma RI$ MØ) $Fc_\gamma RI^-$ was responsible for the majority of the MØ antigen presenting function and PA activity (Table 2). These data lead us to hypothesize that many of the changes seen in patients' MØ function and consequent immunopathology might be attributed to the increase in the $Fc_\gamma RI^+$ MØ subset. We were also able to show that the increased leukocyte pyrogen activity was a result of the increased numbers of $Fc_\gamma RI^+$ MØ in the trauma patient population (Table 3).

Measurement of MØ LP activity was an inaccurate reflection of the MØ cytokine production. It is now known that the MØ can produce IL-6, IL-1, TNF_α, and IL-8, all of which can have leukocyte pyrogen activity. In the next two years of the contract we implemented and adapted a number of assays to distinguish the possible different cytokines produced by MØ. To assay TNF, we adapted the LM bioassay. Since both a cell-associated and a secreted form of TNF_α had been described, we assayed both patient MØ supernates and sonicated cell lysates (32). We found that immunoaberrant patients' MØ produced massively elevated TNF_α levels, but that most of this elevated TNF_α was in the cell-associated, rather than secreted form (Fig.4) (20,22). This observation has recently been independently reported (23).

Another important observation was that the patients' MØ TNF_α production was insensitive to PGE_2 downregulation (Fig.5) (20,22). We had shown that like MØ LP activity, elevated MØ TNF_α levels appeared concomitant to elevated PGE_2 levels. We also showed that addition of exogenous PGE_2 depressed normals' but not patients' MØ TNF_α levels (Table 4). It had previously been shown that normal MØ TNF_α production was both positively and negatively regulated by PGE_2 (33-35). Desensitization of normal MØ TNF_α production to PGE_2 downregulation had been previously described, when unusual or extreme MØ stimulation was introduced (34,35). These data showing that trauma patients' MØ are insensitive to PGE_2 regulation are a further indication that trauma patients' MØ are unusually activated <u>in vivo</u> and deregulated for cytokine production.

One of the unusual stimulations that trauma patients can receive <u>in vivo</u> is crosslinking of their $Fc_\gamma RI$ receptors. Trauma patients have high levels of circulating immunoglobulins and antigen-antibody complexes (36-37). These circulating Ig can stimulate the trauma patients' $Fc_\gamma RI^+$ MØ to produce higher and higher levels of PGE_2 and TNF_α. In addition, we and others have shown that $Fc_\gamma RI$ crosslinking induces MØ production of IL-6 (13,38). This IL-6 can further polyclonally stimulate B lymphocytes to produce more nonspecific immunoglobulins further raising the circulating Ig levels (39,40). Our laboratory, as well as several others, have described early elevations of IL-6 post-trauma (12,13,23,41,42). In addition, we have shown that those trauma patients with elevated MØ IL-6 production also have abnormal numbers of high density $Fc_\gamma RI$ MØ and that it is these $Fc_\gamma RI^+$ MØ which produce the majority of their elevated MØ IL-6 (Fig.6). In fact, these patients' $Fc_\gamma RI^+$ MØ, when stimulated by crosslinking and any other inducer, are the high TNF_α, PGE_2, and IL-6 producers (Fig.7). Our data indicate that the trauma patients' $Fc_\gamma RI^+$ MØ are <u>in vivo</u> activated to produce elevated cytokines, even in the absence of further <u>in vitro stimulation</u>. We hypothesize that the high circulating Ig in trauma patients are induced by IL-6 and that these circulating Ig then further crosslink stimulate susceptible MØ to produce high levels of PGE_2 and TNF_α.

Crosslinking the $Fc_\gamma RI$ receptors on normals' MØ triggers primarily cell-associated TNF_α (Fig.8). A predominance of cell-associated TNF_α is characteristic of the immune aberrant trauma patient and may also be a major contributor to the septic shock syndrome. Cell-associated TNF_α is proposed as being more efficient in delivery of cytotoxic activity than secreted TNF_α (43,44). We have demonstrated that large amounts of cell-associated TNF_α are concomitantly produced in immune aberrant trauma patients. We have investigated a number of mechanisms which might be responsible for elevating patient MØ TNF_α. One such mechanism is an autocrine stimulation of MØ TNF_α. It has been reported that IL-1 and LPS stimulation of MØ downregulates the TNF_α receptors

(TNF$_\alpha$R) preventing the induced TNF$_\alpha$ from autocrine stimulation of the producing MØ (45). As summarized above, Fc$_\gamma$RI crosslinking stimulates TNF$_\alpha$ and appears to be playing a role in the trauma induced stimulation of cell-associated TNF$_\alpha$. Consequently, we examined the effects on TNF$_\alpha$R expression of stimulating MØ with adhesion molecules (matrix adherence), MØ stimulation by Fc$_\gamma$RI crosslinking, bacterial product induction, stimulation with IFN$_\gamma$ and various combinations of these stimuli.

The TNF$_\alpha$R expression was assessed on our EPICS Profile Flow Cytometer using MY9 as our MØ marker and phycoerythrin labeled TNF$_\alpha$ to assess TNF$_\alpha$R expression. In a series of experiments we found that stimulation with MDP and/or matrix adherence downregulated TNF$_\alpha$R expression for about 3 hours. By 18 hours post-stimulation, the TNF$_\alpha$R levels had returned to original levels or were slightly increased (Fig.9). In contrast, stimulation with IFN$_\gamma$ alone or in combination with matrix adherence and MDP caused prolonged downregulation of the MØ TNF$_\alpha$R which persisted during the entire period of production of biologically active TNF$_\alpha$ (Fig.9). These results suggest that MØ actively producing TNF$_\alpha$ are not capable of autocrine stimulation and perpetuating their TNF$_\alpha$ responses. MØ semi-stimulated by matrix adherence and MDP do not produce biologically active TNF$_\alpha$ but are primed to respond to TNF$_\alpha$ in the microenvironment, since the density of their TNF$_\alpha$R has now increased. Interestingly, activating the MØ by crosslinking their 72KD immunoglobulin G receptors (Fc$_\gamma$RI), as would occur when trauma induced circulating immunoglobulins interacted with patients' MØ Fc$_\gamma$RI, has a very different effect on TNF$_\alpha$R expression. Crosslinking MØ Fc$_\gamma$RI activates the cells to produce primarily cell-associated TNF$_\alpha$. Although Fc$_\gamma$RI crosslinking initially downregulates the MØ TNF$_\alpha$R expression, by 18 hours post- stimulation, when the MØ are producing high levels of cell-associated TNF$_\alpha$, the density of TNF$_\alpha$R levels is increased (Fig.10). These data have two implications. First, it is apparent that the cell-associated TNF$_\alpha$ induced by Fc$_\gamma$RI crosslinking is not simply secreted TNF$_\alpha$ bound on the TNF$_\alpha$R as has been sometimes suggested. If that were the case, the TNF$_\alpha$R levels should be decreased on the MØ surface. In actuality, the TNF$_\alpha$R levels are increased. Secondly, these data imply that should cell-associated TNF$_\alpha$ be released or exported, there is potential for MØ autocrine stimulation. This potential may not be great. However, the most provocative findings was that MØ activated by Fc$_\gamma$RI crosslinking and then subsequently stimulated with IFN$_\gamma$ expressed increased densities of their TNF$_\alpha$R during the 18 hour period when they were producing and secreting biologically active TNF$_\alpha$ (Fig.11). If MØ Fc$_\gamma$RI crosslinking preceded IFN$_\gamma$ activation, then the IFN$_\gamma$ no longer prevented expression of high TNF$_\alpha$R levels. The implications of these data for trauma patients is immense. Trauma patients' MØ are frequently Fc$_\gamma$RI activated <u>in vivo</u> in the absence of IFN$_\gamma$. Subsequent challenges which induce IFN$_\gamma$, or perhaps other activators, can now cause an autocrine loop stimulation in which MØ producing biologically active TNF$_\alpha$ will be stimulated to produce more TNF$_\alpha$ and other cytokines by their own TNF$_\alpha$. This may be an important aberrant cytokine stimulation pathway in trauma patients, accounting for some of their increased cytokine levels. We are now assessing changes in TNF$_\alpha$R receptor levels in trauma patients' MØ.

Other mechanisms may also affect patients' levels of cell-associated versus secreted TNF$_\alpha$. Our laboratory has demonstrated a TGF$_\beta$ effect in increasing MØ cell-associated TNF$_\alpha$. As we characterized the aberrant MØ functions of the trauma patient, we noticed that not all of the inhibitory MØ function of the trauma patients could be explained by excessive PGE_2 production. The depressed antigen presenting function of the patients was expressed in their PGE_2 low Fc$_\gamma$RI$^-$ MØ population (Table 5). The depressed APC of the Fc$_\gamma$RI crosslinked MØ could not be reversed by the cyclo-oxygenase inhibitor indomethacin (Indo) (Table 6). The 27KD homodimer TGF$_\beta$ has been repeatedly described as an immunosuppressive product of MØ (45-49). TGF$_\beta$ has immunosuppressive activity for both T cell and B cell proliferation (45). We have demonstrated that immunoaberrant trauma patients' MØ produce elevated levels of TGF$_\beta$ (14) (Fig.12). These abnormally elevated MØ TGF$_\beta$ levels occur late in the post injury period and often correspond to the periods of most elevated cell- associated TNF$_\alpha$ production by MØ. TGF$_\beta$ has been reported to both increase and decrease MØ biological

TNF_α levels (45,46).

In a number of experiments, we have added 2.4ng/ml exogenous TGF_β to both normals' MØ and immunoaberrant trauma patients' MØ. TGF_β addition had little or no effect on the PGE_2 levels produced by normals' MØ. However, addition of exogenous TGF_β to patients' MØ resulted in increased PGE_2 production particularly in those patients whose PGE_2 levels were already elevated (Fig.13). When TGF_β was added to normal MØ in the presence of MDP, small amounts of cell-associated TNF_α were induced, but little or no secreted TNF_α (Fig.14). Addition of TGF_β alone had no effect on normal MØ. However, addition of 2.4ng/ml of exogenous TGF_β to patients' MØ who already had in vivo activated TNF_α production, resulted in a significant increase in MØ cell-associated TNF_α levels. Co-stimulation of patient MØ with TGF_β and MDP produced an even greater augmentation of MØ cell-associated TNF_α levels (Fig.14). MØ secreted TNF_α levels induced by IFN_γ + MDP were actually reduced by TGF_β addition. This dichotomy may explain the conflicting results reported on TGF_β effects on TNF_α production.

These data imply that TGF_β can decrease IFN_γ + MDP induced TNF_α secretion in normals' MØ. However TGF_β can augment TNF_α levels in trauma patients' MØ activated by trauma induced mediators. The implication of this dichotomy for trauma patients is as follows: Trauma patients' MØ are producing elevated TGF_β levels (22). The trauma patient's MØ TGF_β is directly suppressive to T cell proliferation, B cell proliferation and T cell lymphokine production (45-47). This suppressive activity of TGF_β contributes to the immunosuppressed state of the trauma victim. In addition, these elevated post-trauma TGF_β concentrations are present when the patients' MØ are stimulated by other trauma mediators and by bacterial products. The presence of TGF_β during MØ activation alters the subsequent response of patients' MØ to other stimuli. In the presence of TGF_β, MØ PGE_2 responses are increased and MØ TNF_α responses are predisposed toward greatly elevated levels of cell-associated TNF_α. The trauma patients' MØ also seem more sensitive to these TGF_β effects. TGF_β has been shown to stimulate upregulation of its own receptor (46,48). We are currently examining trauma patients' MØ to determine if increased densities of TGF_β receptors post-trauma accounts for their increased sensitivity to TGF_β mediated effects on PGE_2 and TNF_α. The presence of elevated TGF_β levels in the post-trauma microenvironment contributes to the trauma patients' MØ aberrant monokine responses.

TGF_β does not equally augment all monokine production in the trauma patients. IL-6 production does not seem to be increased in the trauma patients' MØ by exogenous TGF_β when TNF_α levels are increased. This again may reflect the MØ activation state (Fig.15). At other post-injury periods when TNF_α is not predominant, we have observed some TGF_β mediated increase in IL-6. As with TNF_α, TGF_β has been reported to both increase and decrease IL-6 levels (45,46). The increased sensitivity of immunoaberrant trauma patients' MØ to TGF_β and the presence of elevated levels of TGF_β will alter the patients' in vivo response to immunotherapeutic modalities administered. For example, treatment of the patients' MØ with IFN_γ altered the effect of TGF_β on TNF_α production, reducing TGF_β's augmenting effect. Since TGF_β is produced in a latent form, the ability of the MØ to activate TGF_β is dependent on their activation state (49). Some of the TGF_β produced by trauma patients is already activated in the culture supernates (14). These data indicate that the trauma patients' MØ are also producing proteolytic enzymes to activate the latent TGF_β they themselves or other cell types produce (49). The availability of activated TGF_β in the microenvironment of trauma patients' MØ is another cause of their altered TGF_β responses.

Development and Adaptation of New Assays to Monitor Functions: As described above, we have adapted the ELISA for PGE_2 measurement, the LM bioassay for secreted and cell-associated TNF_α, the B9, and now MH-60 hybridoma proliferation assay for measurement of IL-6, and the mink lung inhibition assay for assessment of TGF_β. We are currently switching to an ELISA assay for measurement of IL-6. However, no ELISA that we have currently found can accurately measure cell-associated TNF_α. The cell lysates

interfere with the ELISA plate reaction. Since cell-associated TNF is the primary and pivotal product of the immunoaberrant patient MØ, we will continue to use the LM bioassay to detect TNF$_\alpha$ this activity. As described above, we have recently been correlating the appearance of increased TNF$_\alpha$R expression to the increased production of biologically active cell-associated TNF$_\alpha$. In preliminary studies, an increased level of TNF$_\alpha$R on patients' MØ seems to correlate with their increased level of cell-associated TNF$_\alpha$ production. If this correlation holds, fluorocytometric assessment of the patients' MØ TNF$_\alpha$R could be substituted for the LM bioassay as an indicator of cell-associated TNF$_\alpha$ production.

We have also been developing new biological assays to measure two new monokines, macrophage inhibitory factor (MIP-1) and interleukin 8 (50,51). Both these monokines activate neutrophils (50,51). Monocyte produced IL-8 has been suggested as being involved in septic shock (52). In experiments to develop new assays, we have initiated a biological assay for measuring MØ production of PMN activating factors such as MIP-1 and IL-8. Using a fluorescent dye which assesses the production of active oxygen products, we are measuring the effect of MØ supernates on human PMN differentiated from the myeloid leukemia line HL-60. Table 7 illustrates data using this system to detect respiratory burst activity. As can be seen, stimulated respiratory burst activity can be detected using this system.

Assessment of Prophylactic Therapy:

We have also assessed a number of possible different prophylactic therapies over the years. In initial studies, we focused on more broad spectrum immune activators, like TP5, which we hoped would improve the depressed T cell functions. These therapies, however, proved to be less efficient and did not affect the depression to the degree we had hoped. Excessive PGE_2 was a major component of the immunoaberrant trauma patient syndrome and downregulation of MØ PA production was correlated to increases in MØ PGE_2 production (Table 8). We therefore, assessed the effect of indomethacin (Indo) on patient responses. Since we had newly introduced the TNF$_\alpha$ assay to replace measurement of leukocyte pyrogen, we assessed the Indo effect on patients' mitogen responses, MØ APC function, and MØ TNF$_\alpha$ production. The in vitro mitogen responsiveness of the patients' peripheral blood population was slightly improved by Indo addition. However, as already shown in Table 6, the MØ antigen presenting dysfunction was not restored. Even more disturbing were the results from the TNF$_\alpha$ assay.

As we now know, the post-trauma activation state of the trauma patient profoundly affects the way in which trauma patients' MØ respond to subsequent stimuli. As is illustrated in Fig. 16, addition of the cyclo-oxygenase inhibitor indomethacin (Indo), in the absence of an interferon gamma (IFN$_\gamma$) prime, to freshly isolated normals' MØ, only minimally increases their TNF$_\alpha$ production in response to the synthetic gram positive bacterial cell wall analogue muramyl dipeptide (MDP) . In contrast, MØ freshly isolated from patients at 8-10 days post major injury (injury severity score >25), show significant TNF$_\alpha$ production in response to MDP. These patients' TNF$_\alpha$ production is even more exaggerated in the presence of indomethacin (Fig.16). Since, as detailed above, these trauma patients' MØ production of TNF$_\alpha$ is independent of PGE_2 downregulation (20,22), the indomethacin effect must be explained by some mechanism other than a cyclo-oxygenase decrease of PGE_2 levels.

One well described effect of blocking the cyclo-oxygenase pathway is to increase lipoxygenase products' production (53,54) Previous investigators have described a small but significant augmentary effect of LTB_4 on normal MØ production of TNF$_\alpha$ (55,56). We examined the TNF$_\alpha$ augmenting effect of adding 10^{-7} M exogenous LTB_4 to normals' MØ stimulated with 100U of IFN$_\gamma$ + 20μg/ml MDP. Since we assay TNF$_\alpha$ in the LM bioassay, we can simultaneously assess both the TNF$_\alpha$ levels in MØ culture supernates and in the MØ cell lysates (22). All the MØ TNF$_\alpha$ measured is completely neutralizable with anti-TNF$_\alpha$

antibody. We found that LTB_4 does augment MØ TNF_α production and that in some individuals, an enhancement of cell-associated as well as secreted TNF_α levels is observed (Fig.17). This is particularly interesting because the indomethacin enhancing effect on patients' MØ occurs primarily in the cell-associated component of TNF_α activity (Fig.18). In fact, if normal MØ are $Fc_\gamma RI$ crosslink-activated and enriched, the subsequent augmenting effects of LTB_4 on their TNF_α production are greatly increased (Fig.19). As previously shown, $Fc_\gamma RI$ crosslinking increases the induction of cell-associated TNF_α in normals' MØ. Addition of LTB_4 to these $Fc_\gamma RI$ activated normal MØ has a much more pronounced enhancing effect on TNF_α levels than LTB_4 addition to the same MØ without $Fc_\gamma RI$ preactivation. These data again demonstrate that the activation state of the patients' MØ will alter their subsequent response to other mediators or to mediator therapy.

The augmenting effect of LTB_4 on MØ TNF_α production is also independent of any PGE_2 effect (Fig.19). LTB_4 does not enhance all MØ mediator production equally. In those in vivo activated trauma patients with elevated MØ PGE_2 and TNF_α, in vitro addition of LTB_4 further enhances MØ TNF_α and IL-6 levels, but has no effect on MØ IL-1 or PGE_2 stimulation. Normal MØ, which have been $Fc_\gamma RI$ crosslink-activated, also show enhanced IL-6 and TNF_α production when additionally stimulated with LTB_4, but the levels of cytokines they produce are still significantly below those seen in the $Fc_\gamma RI$ activated trauma patients' MØ (Fig.20).

These data suggest the following: first, LTB_4 significantly augments MØ TNF_α induction primarily in unusually activated MØ (i.e. trauma patients or $Fc_\gamma RI^+$ normals); second, LTB_4 enhancement of TNF_α, like the indomethacin effect, primarily affects cell-associated TNF_α; third, MØ PGE_2 levels are not affected by LTB_4 so that reduction in PGE_2 activity cannot account for LTB_4's TNF_α enhancing effect; and fourth, LTB_4 enhances patient $Fc_\gamma RI$ crosslinked MØ more than normal $Fc_\gamma RI$ crosslinked MØ. All of these data are consistent with the hypothesis that the TNF_α enhancing effect of cyclo-oxygenase blockade seen in trauma victims might result from lipoxygenase shunting and increased LTB_4 levels.

The final experiment in this series examined the effect of adding both a cyclo-oxygenase and lipoxygenase inhibitor to $Fc_\gamma RI^+$ activated normals' or trauma patients' MØ. If the indomethacin enhancing effect was the result of lipoxygenase shunting, then simultaneous addition of a lipoxygenase inhibitor should abrogate this enhanced response. In four experiments, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) was added at 40μM to normals' or monokine aberrant trauma patients' MØ. When indomethacin at 10^{-7} M was added along with MDP (bacterial cell wall analogue) to $Fc_\gamma RI^+$ activated normals' or patients' MØ, enhanced production of TNF_α was observed. Simultaneous addition of NDGA to these cultures completely abrogated the indomethacin enhancing effects (Fig.21). These data indicate that the enhancing effect of Indo addition on MØ TNF_α production is due to Indo's augmentation of the lipoxygenase pathway and not to its inhibition of cyclo-oxygenase production. The data also illustrate again the greatly elevated TNF_α response of trauma patients' MØ and that subsequent stimulation of patients' intensely activated MØ leads to exaggerated responses.

The prophylactic use of indomethacin to block the immunosuppressive effect of PGE_2 post-trauma would only be possible if combined with administration of a lipoxygenase inhibitor. These data illustrate the importance of evaluating prophylactic therapy on trauma patients' cells in vitro, as well as using animal models. As yet, there is no model with the ability to duplicate the exact activation state of post injury cells. An endotoxin bolus model does not produce this same level of interrelated MØ activation. In addition, there is a problem with some rodent models not being at all parallel to humans for cytokine evaluation. One such cytokine is IL-4.

We originally explored the effects of IL-4 as an activator of T cell function.

Il-2 had been shown to be somewhat effective in increasing T cell function in trauma patients. We, therefore, assessed IL-4's ability to augment T mitogen responses, since it was reported to increase T cell function (57). In addition, IL-4 was reported to upregulate murine monocyte function, so we examined its effect on MØ APC (58,59). We also examined IL-4's effect on MØ TNF_α and PGE_2, since we were concerned that increasing the MØ function might also increase MØ TNF_α production. Much to our surprise, IL-4 , although not signigicantly increasing MØ APC function, significantly decreased MØ TNF_α and PGE_2 activity (60). IL-4 has now been shown to downregulate some human MØ functions while potentiating others (61-64). Human IL-4's activity on human MØ appears quite different than murine IL-4's activity on murine macrophage (58,59). IL-4 has been shown to downregulate human MØ production of IL-1, TNF_α, IL-6 and PGE_2 in response to LPS stimulation (58,64).

We examined the ability of exogenously added IL-4 to downregulate aberrant mediator production by patients' MØ. Patients' or normals' MØ stimulated with MDP have increased PGE_2 activity. As can be seen in Fig. 22, patients' MØ were in vivo preactivated and produced immunosuppressive levels of PGE_2 (>35ng/10^6 MØ). Stimulation with MDP further elevated the MØ PGE_2 activity. Addition of IL-4 over a concentration range of 0.5ng/ml - 25ng/ml downregulated the patients' MØ PGE_2 levels in a dose-dependent fashion. Addition of 5-25ng/ml of IL-4 decreased PGE_2 levels to the non- immunosuppressive normal range (<35ng/10^6 MØ). Simultaneously assessed normal MØ's PGE_2 levels were also reduced by IL-4 addition (Fig.22). Interestingly, addition of IL-4 to patients' MØ in the same concentration range (5-25ng/ml) reduced their TNF_α production but did not return it to normal levels. (Fig.23). As can be seen in Fig. 23, patients' MØ were producing considerable cell-associated TNF_α due solely to their in vivo stimulation. Further stimulation of patients' MØ with MDP induced production of additional secreted TNF_α. MDP does not induce significant secreted TNF_α in normals' MØ if an IFN_γ prime is not co-present. Addition of a suboptimal prime with IFN_γ (10U/ml) induced a significant secreted TNF_α response in both the patients' and normals' MØ. The patients' MØ also increased their cell-associated TNF_α production while normals' MØ produced no cell-associated TNF_α (Fig.23). Addition of 5ng/ml of IL-4 downregulated the normals' IFN_γ + MDP induced TNF_α response by >65%. In contrast, 25ng/ml of IL-4 was only able to downregulate patients' secreted TNF_α by 50% and their cell-associated TNF_α by 50%. Patients' MØ TNF_α levels were still abnormally elevated even in the presence of 25ng/ml of IL-4. These data imply that IL-4 can downmodulate patients' aberrant monokine responses, but that the effective dose required for control of TNF_α induced septic shock might be unrealistic for a clinical situation. A combinational therapy of IL-4 plus other immune modulators might be more effective in controlling the aberrant TNF_α levels of significantly immunoaberrant trauma patients.

We have also examined IL-4's ability to downregulate excessive patients' MØ IL-6 production (65). As can be seen in Fig. 24, patients' excessive IL-6, which is produced primarily by the $Fc_\gamma RI$ crosslinked MØ, can be downregulated by IL-4. This IL-4 mediated downregulation occurs concomitant to downregulation of the patients' excess MØ PGE_2 production. These data suggest that IL-4 could be effective immunotherapy in cases when TNF_α levels were not massively elevated. Particularly, early in the post-trauma period, IL-4 might be useful.

Finally, we examined IL-4's effect on TGF_β levels. Here we have been able to show that MDP induced TGF_β levels in normals' MØ can be downregulated by IL-4 (Fig.25). As a cautionary note, low levels of IL-4 sometimes seem to augment TGF_β production. Consequently, we need to examine IL-4's effect on patients' TGF_β levels before making any conclusions on the IL-4 effects on patient TGF_β production.

In another set of experiments, we examined synthetic glucans as possible prophylactic mediators post-trauma. Early reports had indicated a positive effect of glucan in some septic models (66). We did not become interested in glucan as a possible immunomodulator until it was reported to decrease MØ PGE_2 levels post-trauma

(67). At that time, a commercial developer of synthetic glucan offered to provide glucans to our laboratory for in vitro testing. We were particularly concerned as to whether this synthetic glucan would activate patients' MØ TNF_α production. The advantage of the synthetic glucan was that as an engineered product, it is endotoxin free. The original disadvantage was that the material was particulate in nature. Consequently, there is some phagocytic stimulation of the patients' MØ. As can be seen in Table 9, addition of .15μg/ml of particulate glucan to patients' MØ downregulated their PGE_2 response. However, the TNF_α results were more variable. Some of the variability was due to the phagocytic stimuli caused by the glucan particulate nature.

In 1990, a soluble form of the glucan became available. When we assessed the soluble glucan effect, we were pleasantly surprised. As before, we were particularly concerned with the effect of adding glucans to trauma patients' MØ with elevated TNF_α levels. Previous reports had suggested that glucan types of stimuli (i.e. zymosan) downregulated normals' MØ PGE_2 production by increasing their LTB_4 production. Since we have produced evidence (summarized above) that LTB_4 is an augmentor of TNF_α levels in previously trauma-activated MØ, we were concerned that soluble synthetic glucans (PGG) might increase patients' TNF_α levels. As can be seen in Fig 26, soluble PGG, added at a concentration of 20μg/ml, was able to downregulate MØ PGE_2 production by both trauma patients' and normals' MØ. In Fig. 27, the data illustrates that 20μg/ml of PGG also slightly downregulates patients' MØ TNF_α levels, rather than increasing these levels. The level of PGG downregulation is not statistically significant for TNF_α. However, we are now trying higher PGG doses, 25μg - 30μg/ml to see if we can get more significant effects on TNF_α. We also examined the effects of soluble glucans on MØ IL-6. We found that when very high levels of IL-6 were already being produced, then glucan at 20μg/ml had no downregulating effect (Fig.29). However, if interferon γ was given as an additional in vitro stimuli to trauma patients' MØ, then we sometimes saw an increase in IL-6 levels. This is somewhat troublesome. However, trauma patients are known to have highly depressed IFN_γ production and high IL-6 levels are reported to decrease TNF_α levels. We tried this combination therapy (i.e. PGG + interferon γ) because many investigators have suggested IFN_γ as a possible prophylactic in trauma patients. In addition, the combination of PGG + IFN_γ is more effective in downregulating patients' MØ PGE_2 levels but augments patients' MØ IL-6 levels (Fig.26). Here again, however, a potential therapy (i.e. IL-6 and IFN_γ) that appears promising in some animal models does not appear to be as beneficial in trauma patients whose MØ are already highly in vivo activated. Nevertheless, glucan therapy, in combination with other modulators (like IL-4), may have high potential for benefiting trauma patients. In fact, in a recent publication, in vivo administration of glucan to trauma patients, was shown to decrease serum PGE_2 levels without increasing serum TNF_α levels. Although no decrease in TNF_α levels were seen, neither was a correlation of serum TNF_α levels to sepsis seen (6). It is our contention that the cell-associated TNF_α levels are more critical in post-trauma septic syndrome and our in vitro data indicate that post-trauma cell-associated TNF_α is slightly decreased by glucan treatment. Our data indicate that commercially available synthetic glucan may have prophylactic benefits in combat casualty care.

Conclusion:

In summary, during this contract period we have made substantial progress in characterizing those dysfunctions which are most critical in the post-trauma septic syndrome. In addition, we have defined the interactions of these aberrant MØ functions. We have adapted a number of new assays for specifically assessing patients' MØ functions. We have also explored a number of possible prophylactic therapies.

During this period, we have shown that immunoaberrant trauma patients' MØ produce abnormally elevated levels of IL-6, PGE_2, TGF_β, as well as TNF_α. These patients' MØ TNF_α are insensitive to PGE_2 downregulation and largely consist of cell-associated

TNF_α. The in vivo activated MØ from these trauma patients are aberrant in their response to the cyclo-oxygenase inhibitor indomethacin in that their TNF_α levels are abnormally augmented by Indo. These patients' abnormal increase in MØ TNF_α levels in response to Indo appear to be a result of increased sensitivity to stimulation by lipoxygenase products, particularly LTB_4. The patients' trauma activated MØ are not just more sensitive to LTB_4 but also to other trauma induced mediators in the microenvironment. Crosslinking the patients' MØ $Fc_\gamma RI$, as would occur in vivo by excessive circulating immunoglobulin, results in exaggerated production of TNF_α, IL-6 and PGE_2 by patients' MØ. We have also shown that immunoaberrant patients' MØ also have increased sensitivity to TGF_β. Excessive MØ produced TGF_β is available in the post-trauma microenvironment (14). This TGF_β is activated from its latent form by MØ produced proteolytic enzymes (49). The post-trauma MØ is now autocrine stimulated by its own TGF_β. The post-trauma activated MØ produces both greater PGE_2 activity and augmented levels of cell-associated TNF_α in response to the activated TGF_β in its microenvironment. The normal unactivated MØ is unaffected or even depressed by exogenous TGF_β. The PGE_2 and TNF_α responses of normal MØ that have been preactivated by $Fc_\gamma RI$ crosslinking and then exposed to TGF_β parallels the patients' MØ responses in kind but not quantity.

The response of trauma-activated patients' cells is very different from normals' and leads to questions about the actual response of patients' MØ to in vivo therapy. We have examined indomethacin, lypoxygenase inhibitors, synthetic glucans and interleukin-4, as possible immunomodulators post-trauma. Indo, as described above, was effective in PGE_2 downregulation, but massively increased MØ cell-associated TNF_α levels. The addition of a lypoxygenase inhibitor prevented this TNF_α augmentation, but had no effect on post-trauma MØ PGE_2 levels. Consequently, a combinational therapy of Indo and a lypoxygenase inhibitor may be indicated. Synthetic glucans would decrease post-trauma MØ PGE_2 levels and TNF_α levels, but had no effect on MØ IL-6 production. This inexpensive drug might have broad usage in post-trauma prophylactic therapy. IL-4 was effective in downregulating patients' elevated MØ PGE_2 activity, but was less effective in correcting the patients' MØ aberrant TNF_α responses. The unusual activation of trauma patients' MØ by trauma-generated mediators like complement split products, circulating immunoglobulin and fibrin degradation products etc., results in a preactivated monocyte. The subsequent response of this in vivo activated MØ to bacterial stimuli or other trauma-generated mediators varies greatly from that of normals' monocytes. These altered MØ response potentials can be predicted by assessing the trauma patients' MØ in vitro for their response to potential mediators or immunotherapeutic modalities.

This contract has met all the goals set for it and expanded the information available on the treatment and pathology of trauma patients. This data is directly applicable in the care of combat casualties and should aid in the Army's mission of the best care and early return to duty for its members.

Table 1. Trauma Increases Some MØ Functions While Decreasing Others.

Cell	LPΔtemp[a]	ΔPA%[b]	ΔPGE_2[c]
Norm MØ	.15	59.5	6.8
Pt MØ	.35 → .65	41.8 → 14.1	1.6 →23.6

[a] Leukocyte pyrogen (LP) was assessed as change in temperature of mice injected with MØ supernates. The patient (pt) data represent initial to maximum values. The normal (norm) data represents the paired normal at the time of patient maximum change.

[b] MØ production of plasminogen activator (PA) is measured in percent PA specific fibrinolysis.

[c] Prostaglandin E_2 in programs per 10^6 recovered MØ.

TABLE 2. FcRI⁻ MØ Subset Has Greater Activity in Plasminogen Activator Production and Antigen Presentation Capacity

	Plasminogen activator (% specific fibrinolysis)[a]			Antigen presentation (cpm)		
	Exp. 1	Exp. 2	Exp. 3		Exp. 4	Exp. 5
FcRI⁺[b]	19.0	36.1	23.7	FcRI⁺[c]	9.165	10.437
FcRI⁻	57.2	51.4	35.5	FcRI⁻[c]	52.637	35.824

[a] MØ plasminogen activator production was measured as described in "Materials and Methods."

[b] FcRI⁺ and FcRI⁻ MØ subsets were separated by rosetting the MØ with anti-RH-coated human erythrocytes as described in "Materials and Methods."

[c] FcRI⁺ and FcRI⁻ MØ were pulsed with antigen (tetanus toxoid) in the antigen presentation assay. After the removal of excess antigen, antigen-pulsed MØ were co-cultured with syngeneic T cells.

Table 3. Fc⁺ MØ Subset Contains the MØ which are Producing Leukocyte Pyrogen (LP)

MØ Pop[A]	Patient		Normal	
	PGE_2	LP[B]	PGE_2	LP
Fc^+	12,665	.55	4,058	.25
Fc^-	1,484	0	847	0

A. MØ populations (Pop) were selected for Fc receptor by rossetting with anti RH coated red cells.

B. Leukocyte pyrogen was assayed as change in temperature (ΔTemp) of mice injected with 0.3 ml of MØ supernate.

Table 4

Suppressed[a] Trauma Patients' MØ TNF_α Production is Insensitive to PGE_2 Downregulation

		TNF_α[b] ng/10^6 MØ		PGE_2 ng/10^6 MØ	
		10γ[c] +MDP	10γ +MDP+PGE_2	10γ +MDP	10γ +MDP+PGE_2
Suppressed	Pt	5.2/6.4	17.5/0	41.5	142.7
	Nor	0/6.3	0/0	8.2	118.3
Suppressed	Pt	16.1/.3	15.9/0	29.7	60.0
	Nor	0/0	0/0	9.1	- -
Suppressed	Pt	4.1/14.6	15.8/.24	29.1	184.5
Immcomp[d]	Pt	3.8/1.3	0/0	11.5	79.1
	Nor	0/2.4	0/0	12.9	89.6

a. Suppressed trauma patients were those with PGE_2 levels >25

b. TNF_α presented as cell associated/secreted

c. γ+MDP = 10U of gamma interferon + muramyl dipeptide

d. Immunocompetent patients

Table 5: Depressed APC capacity of pt MØ
(^{3}H-TdR cpm)

	Normal	Immunosuppressed Patient
$FcRI^+$	1,764	1,335
$FcRI^+p^1$	1,769	857
$FcRI^-$	5,406	1,191
$FcRI^-p$	14,618	2,378

1. MØ pulsed(p) 24 hrs with Tetanus Toxoid

Table 6 : Inability of $FcRI^+$ MØ to act as APC isn't primarily due to excessive PGE_2

MØ Treat	Exp40	Exp37
$FcRI^+p^1$	9,165	10,437
$FcRI^+p$+Indo	9,213	5,625
$FcRI^-p$	52,637	35,824
$FcRI^-p$+Indo	55,220	32,152

1. MØ pulsed(p) 24 hrs with Tetanus Toxoid

Table 7

	UNDIFFERENTIATION (HL-60)		DIFFERENTIATION[a]-HL-60 (PMN)	
	Unstimulated	Stimulated (PMA)[b]	Unstimulated	Stimulated (PMA)
Experiment 1:	2.84[c]	3.39	51.22	192.50
Experiment 2:	—	—	52.63	164.10

a. Differentiation of HL-60 to PMN was accomplished by 24 hour induction with Retinoic Acid (10^{-6}M) and Di Methyl Formamide (60mM) for 72 hours.

b. Differentiation of HL-60 were stimulated for 30 minutes at 37°C with Phorbal Myacetate (100 μg/ml).

c. Data is represented as mean fluorescent intensities.

Table 8

EFFECT OF MDP[A] ON NORMAL OR PATIENT MO FUNCTION

Mo	PGE_2[b]	PA[c]
Norm	16 944 ± 6 717	31.4 ± 7.4
Norm + MDP	54 387 ± 23 030	15.2 ± 3.8
D1 Pt	19 759 ± 7 295	33.5 ± 12.1
D1 Pt + MDP	49 049 ± 22 529	13.8 ± 4.2
D>6 Pt	37 940 ± 11 606	14.4 ± 4.1
D>6 Pt + MDP	91 851 ± 20 951	14.0 ± 1.6

A. $2x10^6$ MO CULTURED FOR 2 DAYS WITH 20 /UG/ML OF MURAMYL DIPEPTIDE (MDP)

B. PROSTAGLANDIN E2 PRODUCTION IN PICOGRAMS PER 10^6 RECOVERED MO

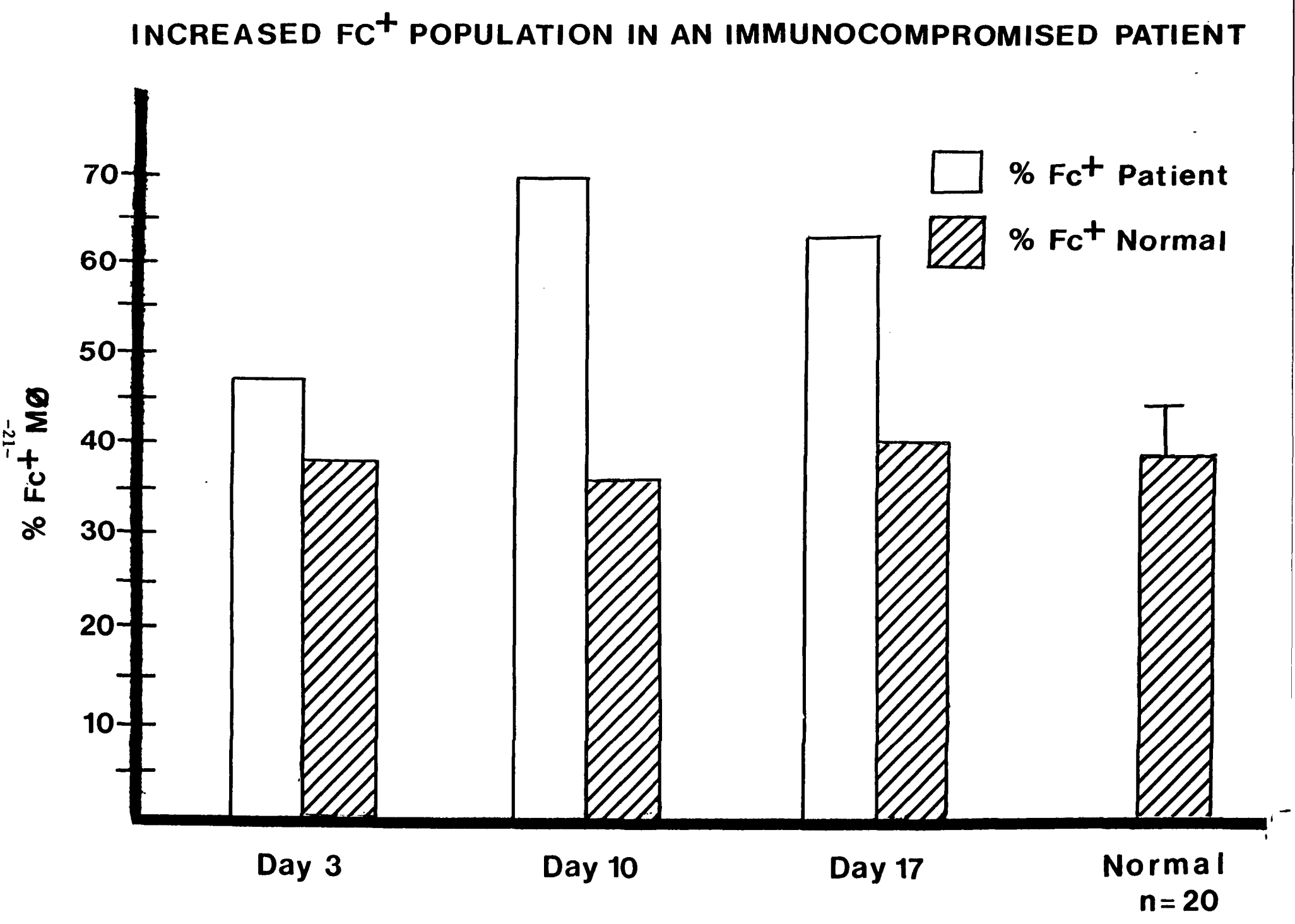
C. PLASMINOGEN ACTIVATOR ACTIVITY IN % SPECIFIC FIBRINOLYSIS.

Table 9

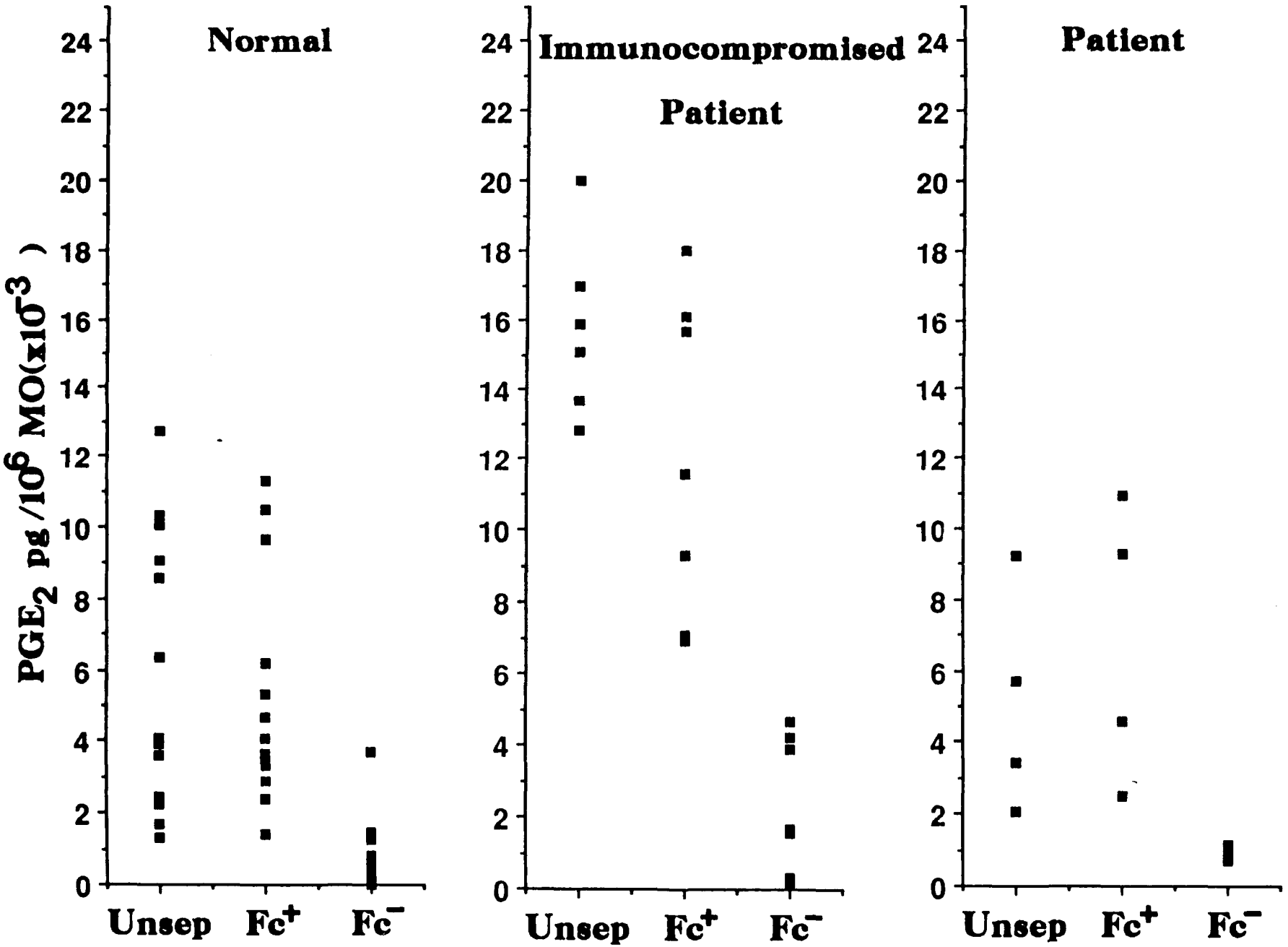
Downregulation of MDP induced elevated PGE_2 levels by glucans in patients' monocytes[a]

	Patients		Normal	
	PGE_2 ng/ml	% downregulation	PGE_2 ng/ml	% downregulation
medium	4.80	-	16.23	-
MDP	33.75	-	15.85	-
MDP + B	7.54	78%	2.30	85%
MDP + R4	6.75	80%	3.12	80%
B WGP	3.01	37%	2.06	87%
R4 WGP	1.18	75%	1.91	88%

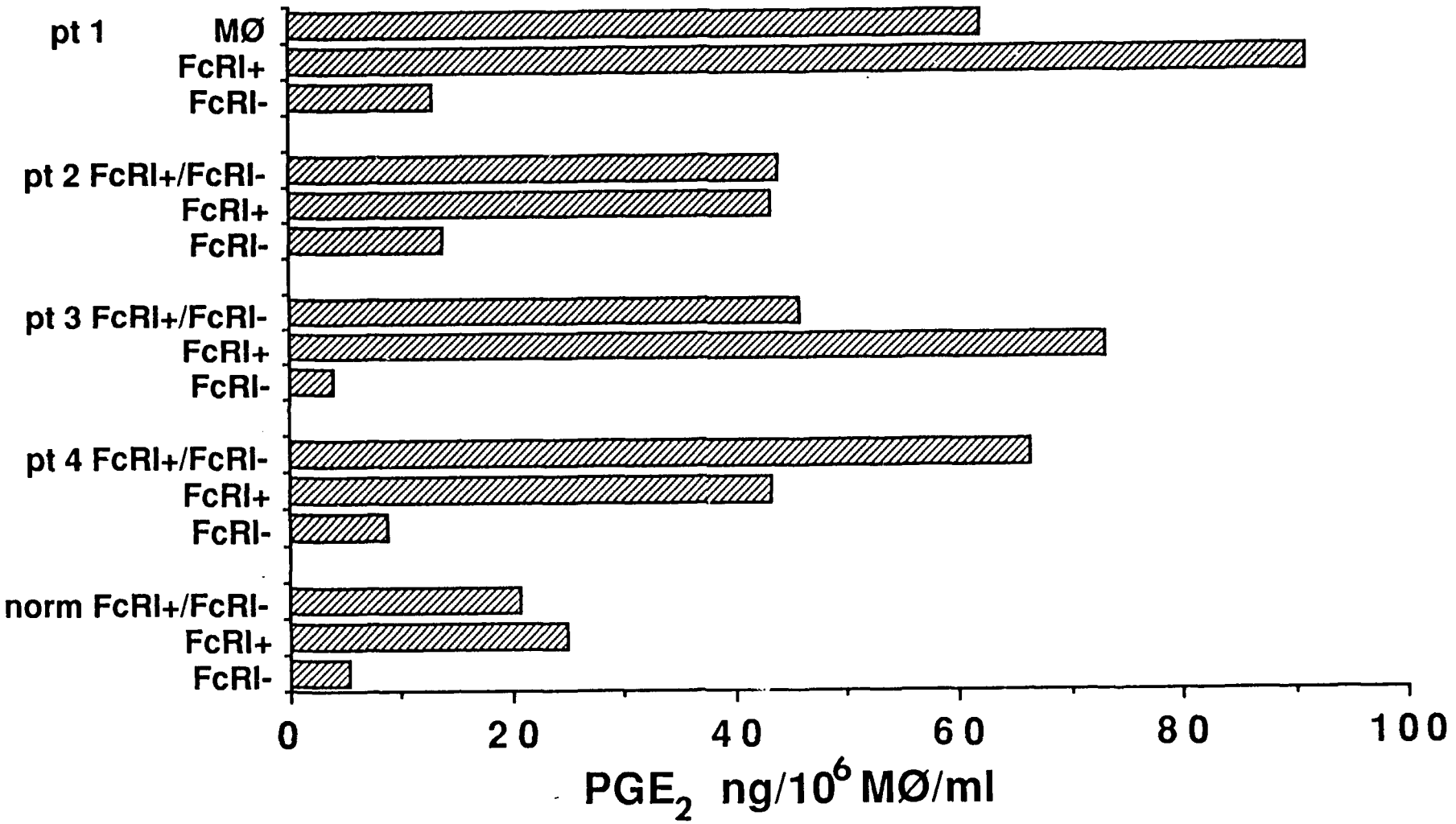
a. MØ PGE_2 levels were measured after stimulation with 20μg/ml MDP, 15μg/ml B WGP, 15μg/ml R4 WGP or with their combinations as indicated for 16 hours.


INCREASED FC+ POPULATION IN AN IMMUNOCOMPROMISED PATIENT
% Fc+ Patient
% Fc+ Normal
% Fc+ MØ
70
60
50
40
30
20
10
Day 3
Day 10
Day 17
Normal
n=20

THE FC^+MØ SUBSET OF IMMUNOSUPPRESSED PATIENTS PRODUCES THE MAJORITY OF THEIR ELEVATED PGE_2



Stimulation by Crosslinking FcRI Induces Greater PGE_2 Levels in Patients' Versus Normals' MØ



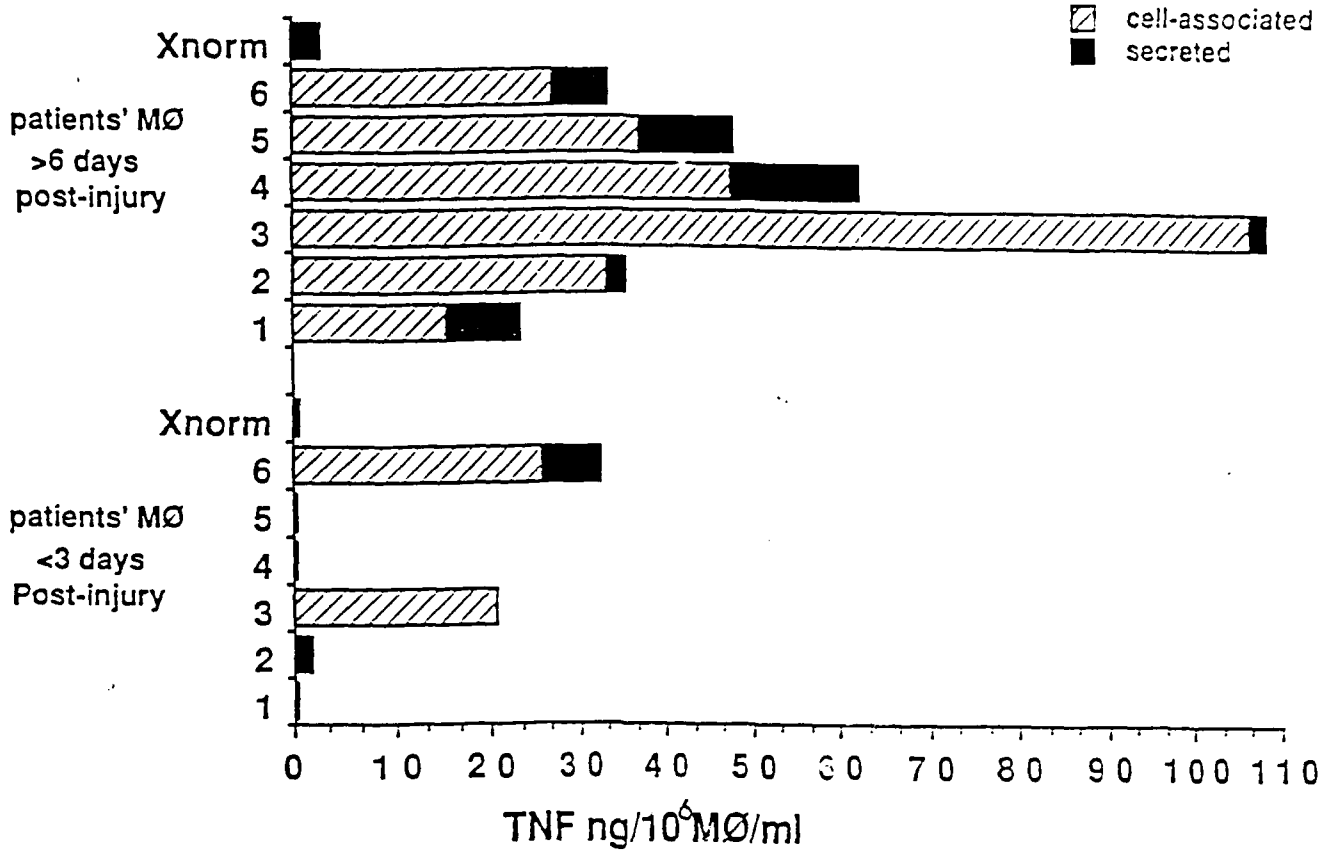


FIG. 4. Elevated cell-associated TNF_α in 6 representative immunosuppressed trauma patients. TNF_α levels of MØ supernates (secreted) or cell lysates (cell-associated) as assayed in LM bioassay. All TNF_α activity is neutralizable with anti-TNF_α antibody. Median of normals assayed simultaneously with patients after suboptimal 10 U IFN, and 20 μg/ml MDP stimulation.

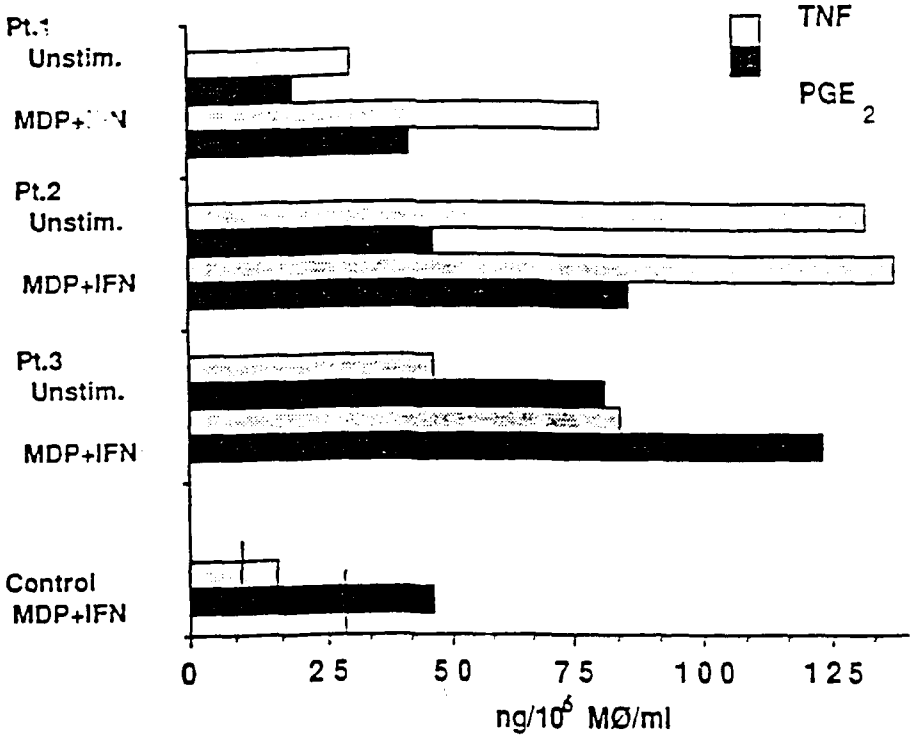
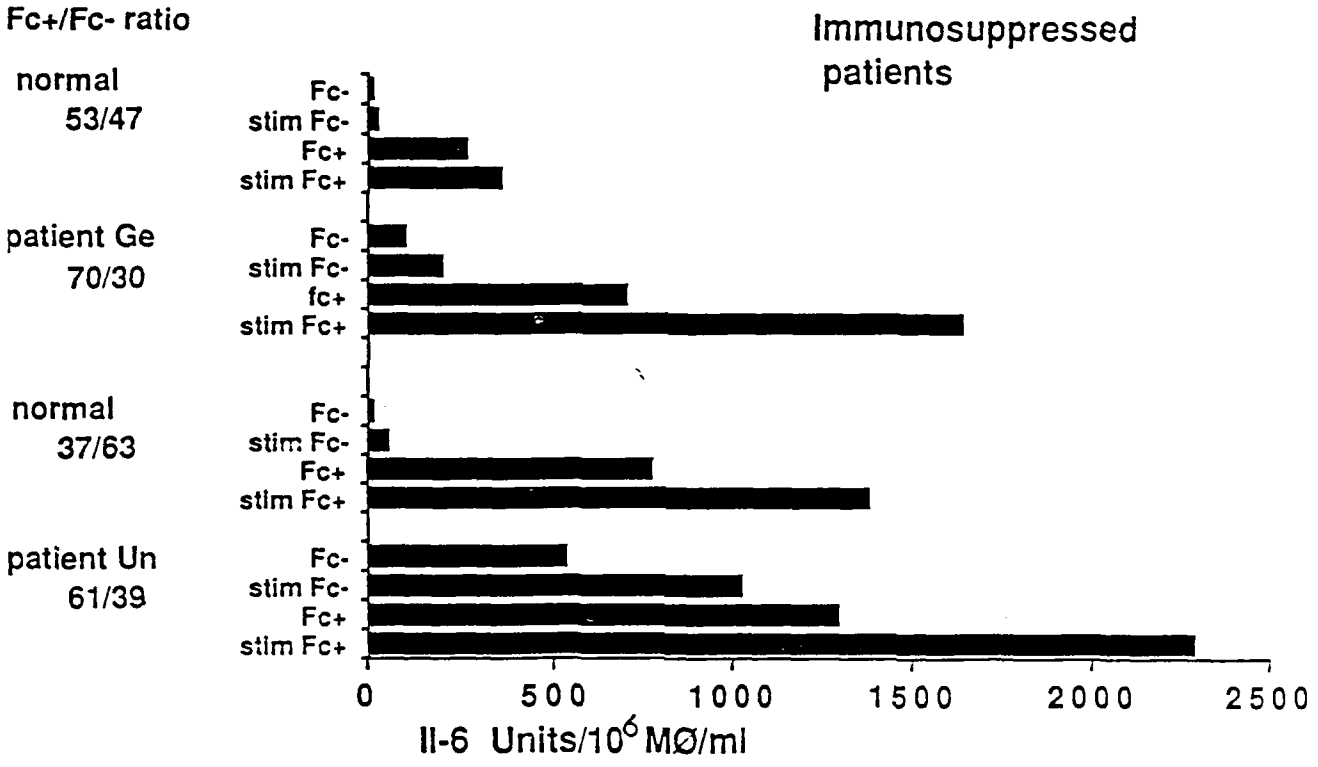


FIG. 5 Concomitantly increased MØ TNF_α and PGE_2 in immunosuppressed trauma patients. Data from 3 representative patients' monocytes with no in vitro stimulation or stimulated with suboptimal (10 U interferon $_\alpha$ + 20 μg MDP) were assayed for TNF_α (LM bioassay) and PGE_2 (ELISA) levels. TNF_α and PGE_2 values are nanograms/10^6 MØ/ml. Only normal MØ response to suboptimal stimulation is shown as median with range. Patient values (n = 23) were statistically increased over normal, p = <0.001.



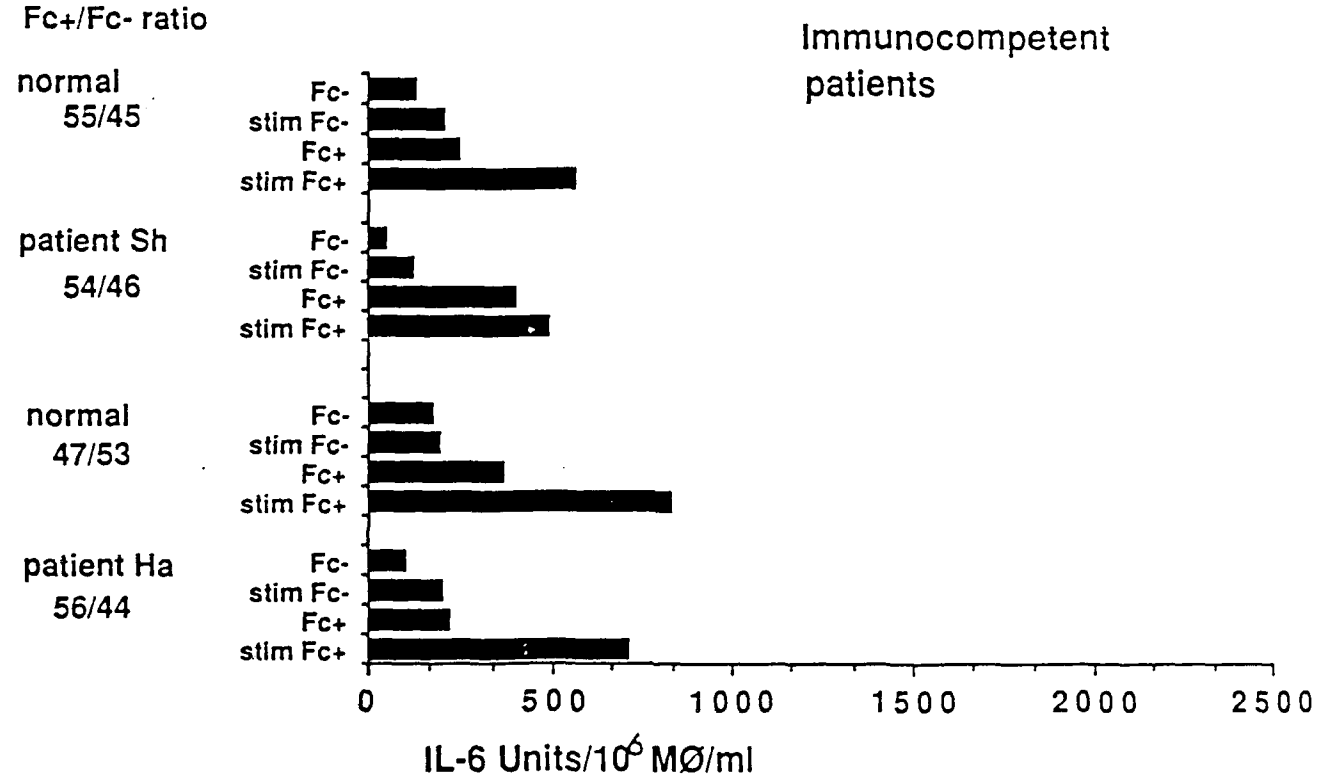
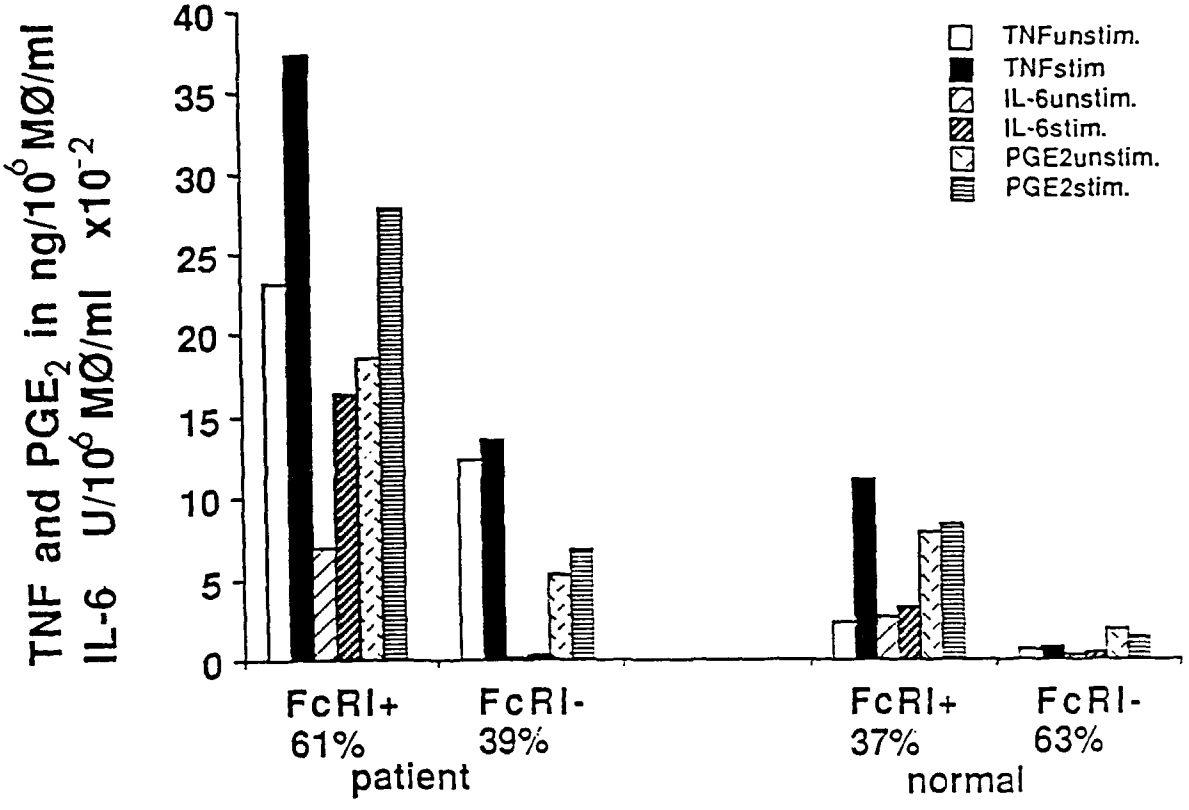


FIG. 6 Trauma patients with increased proportions of $FcRI^+$ MØ have elevated MØ IL-6 levels. Patient and normal MØ were isolated, the $FcRI^+$ separated, and the ratio of $FcRI^+$ to $FcRI^-$ MØ determined. Medium normal ratios of $FcRI^+$ to $FcRI^-$ is 40/60. MØ were stimulated with 10 U IFN, + 20 μg/ml MDP and their IL-6 production assessed in a hybridoma (B9) bioassay.



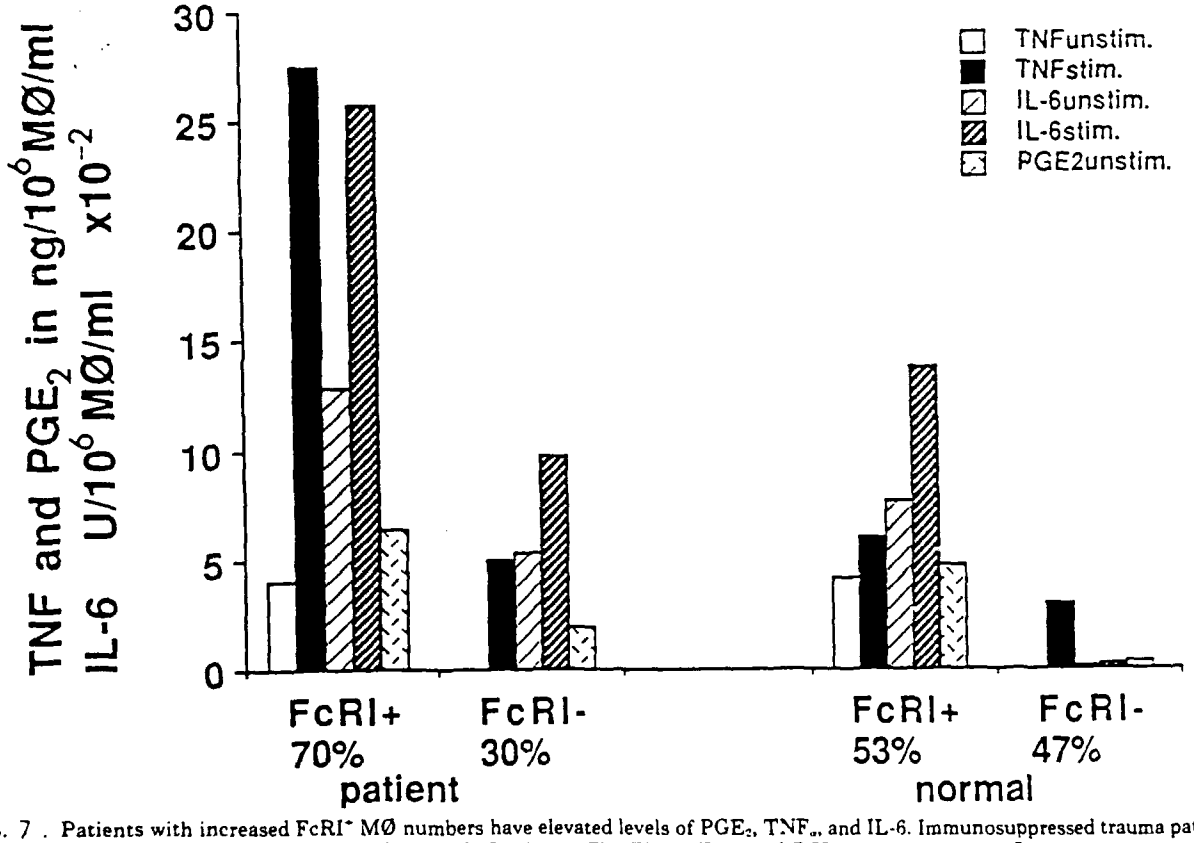
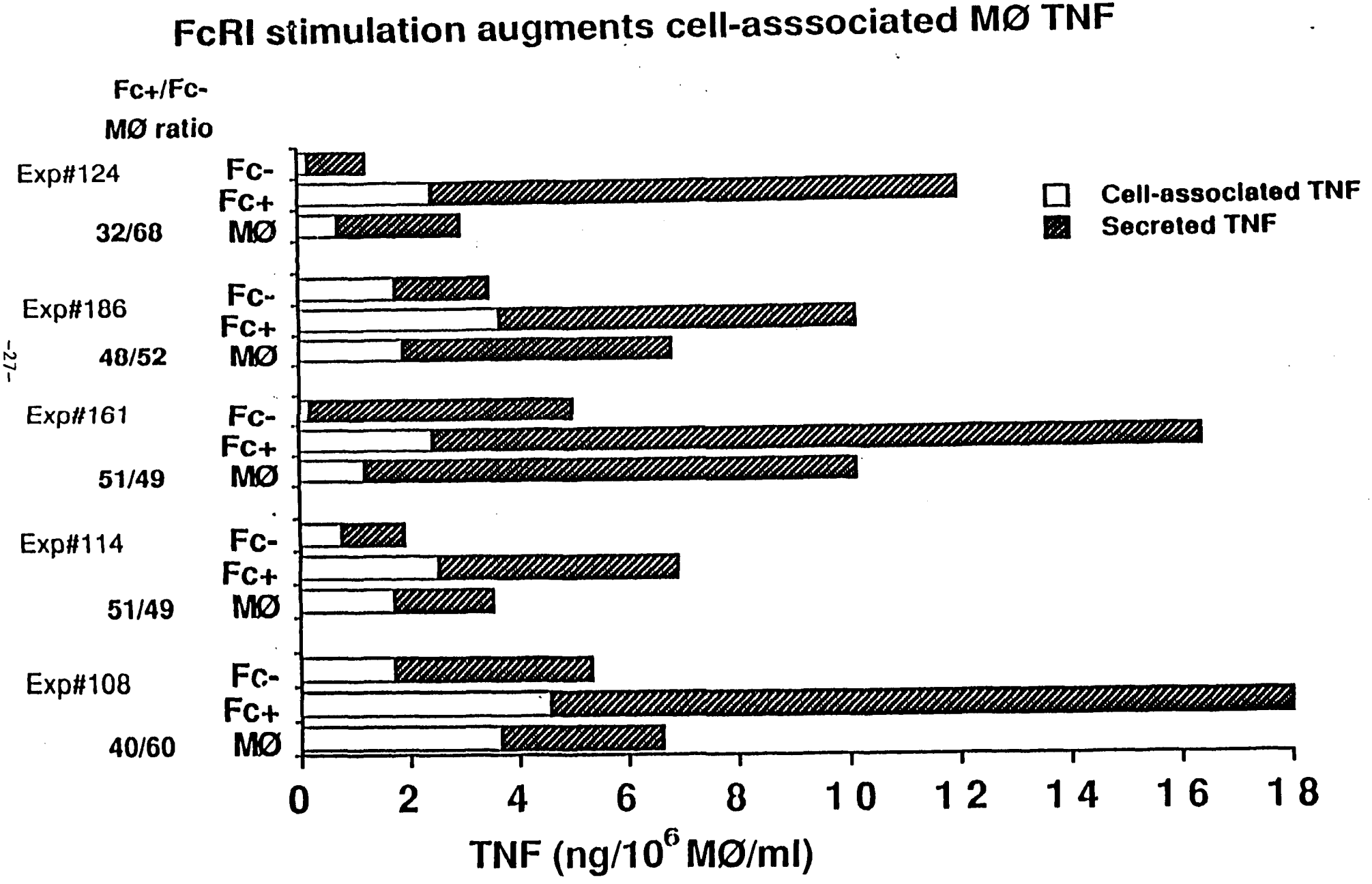
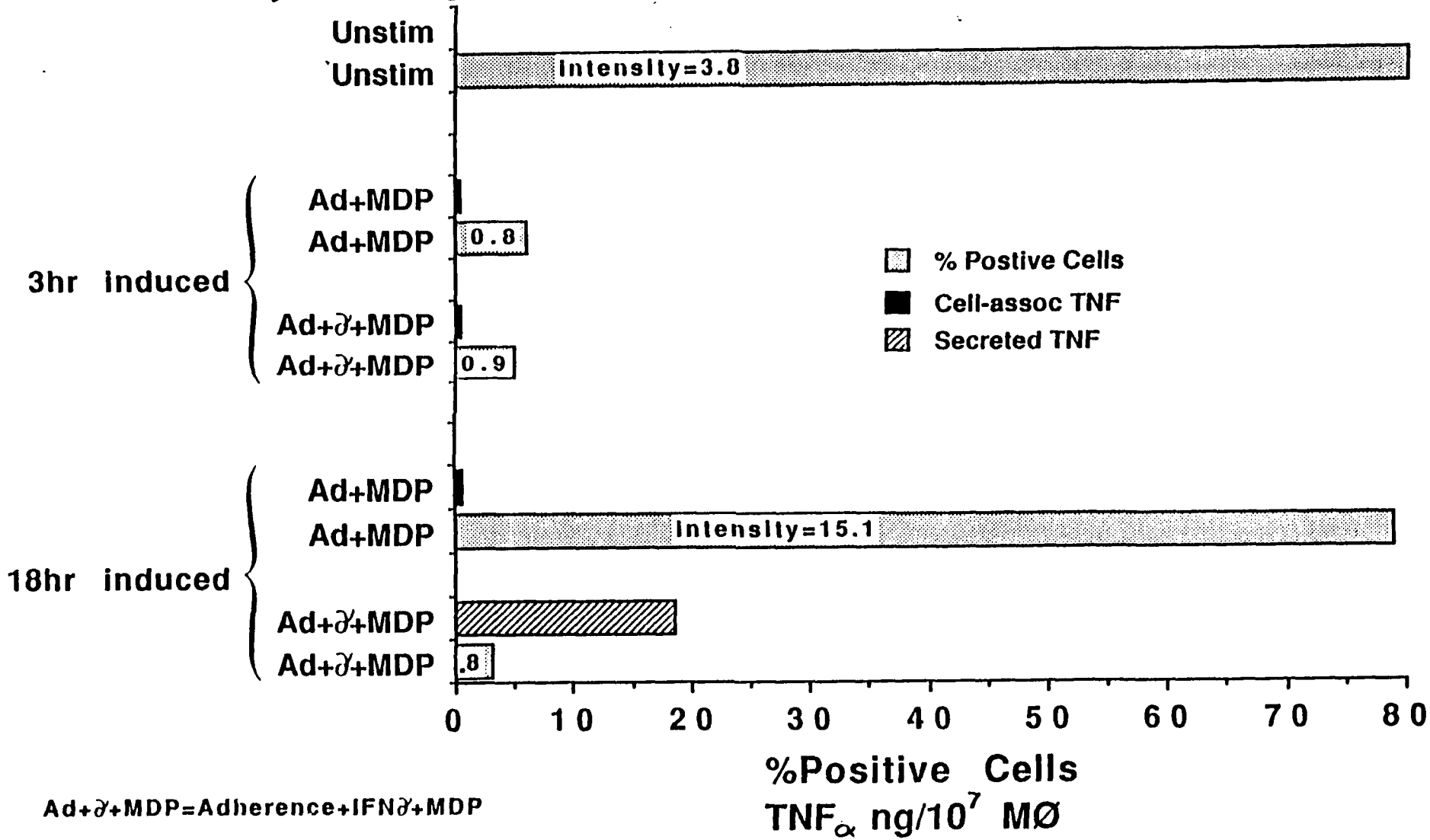


FIG. 7 . Patients with increased FcRI$^+$ MØ numbers have elevated levels of PGE_2, TNF_α, and IL-6. Immunosuppressed trauma patients' and normals' MØ were segregated into FcRI$^+$ and FcRI$^-$ MØ subsets. The TNF_α, IL-6, and PGE_2 levels of these MØ populations were assayed as described and compared. MØ were stimulated with IFN_γ + MDP (top panel) or indomethacin (10^{-6}M) and MDP (bottom panel). Per cent of FcRI$^+$ or FcRI$^-$ in total MØ population shown at bottom.

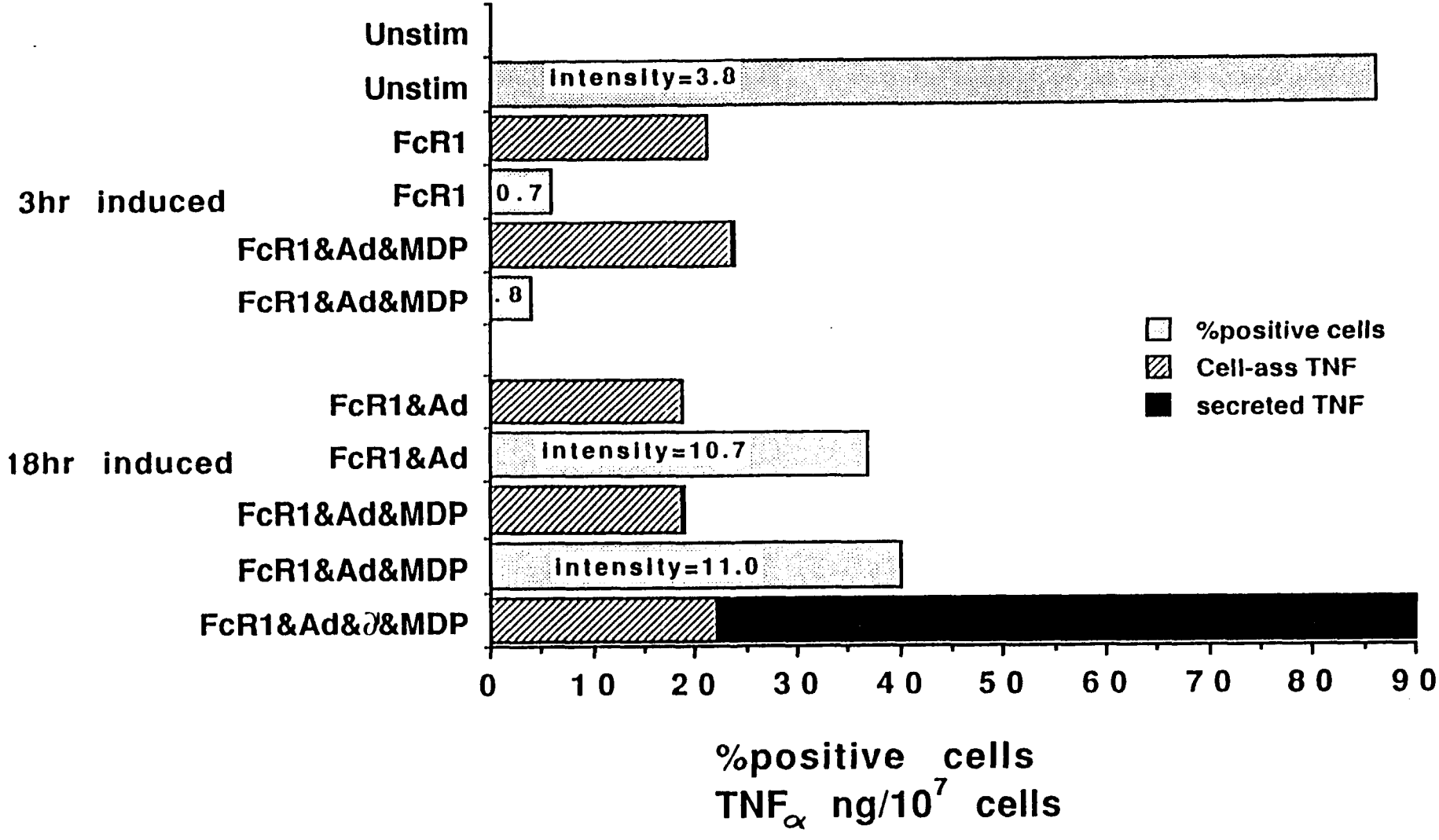

FcRI stimulation augments cell-asssociated MØ TNF
Fc+/Fc-
MØ ratio
Exp#124
32/68
Exp#186
48/52
Exp#161
51/49
Exp#114
51/49
Exp#108
40/60
Fc-
Fc+
MØ
Cell-associated TNF
Secreted TNF
0
2
4
6
8
10
12
14
16
18
TNF (ng/10^6 MØ/ml)

When IFN_γ +MDP induces secreted TNF_α activity, MØ TNFR expression is continuously downregulated while MDP alone upregulates TNFR levels

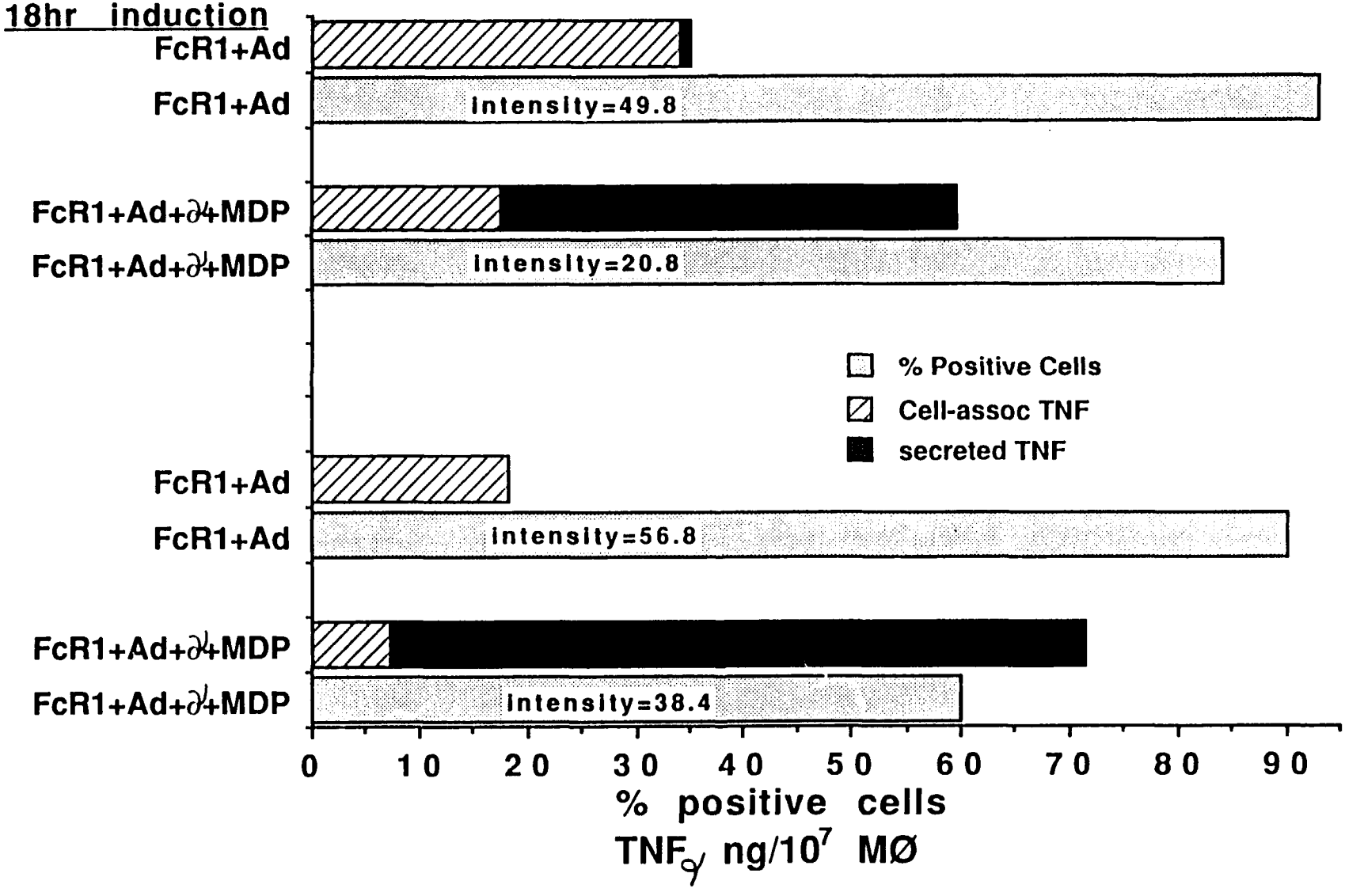


Ad+∂+MDP=Adherence+IFN∂+MDP

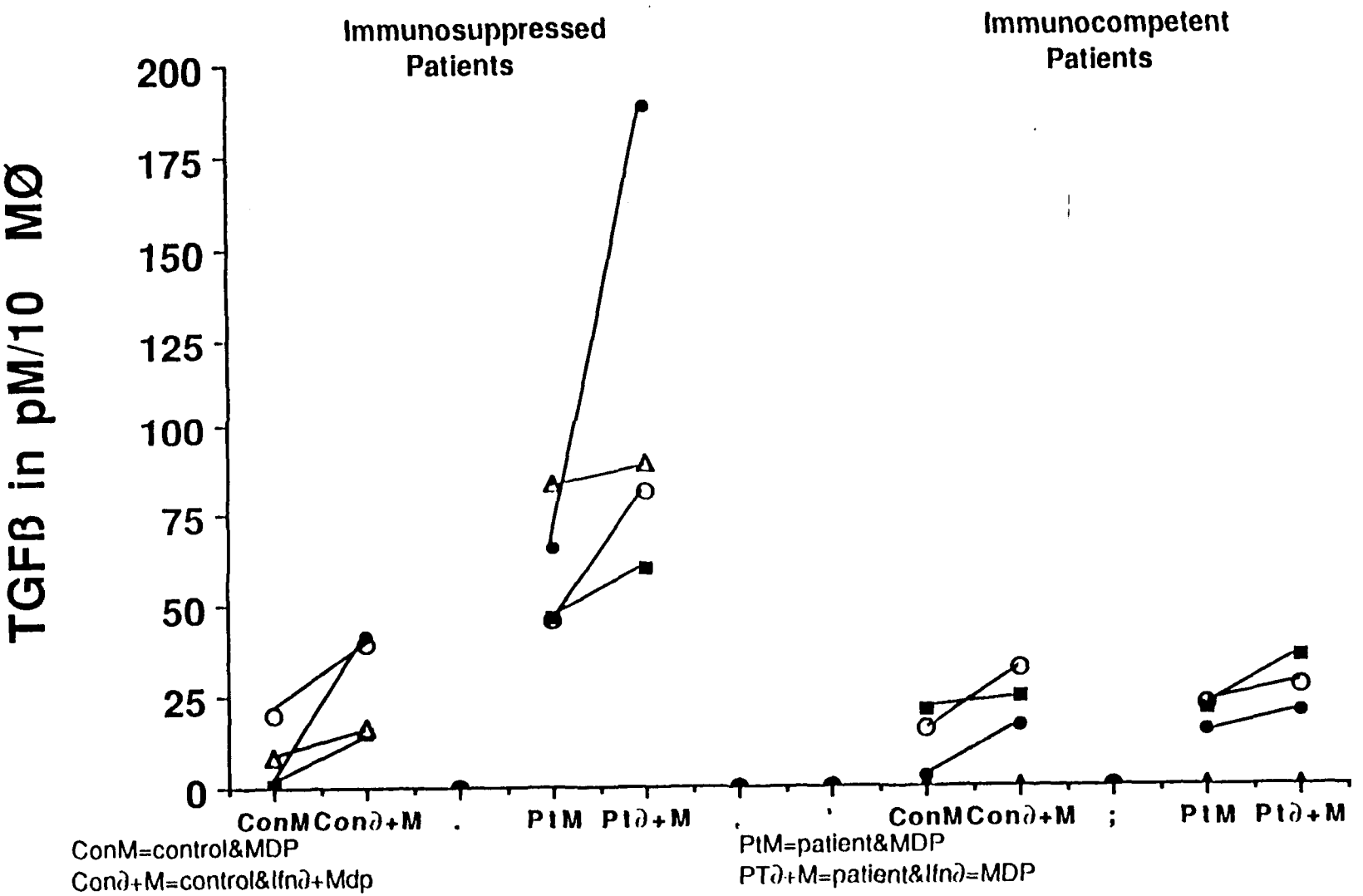
$Fc_\gamma R1$ crosslinking does not continue to downregulate TNFR expression during cell-associated TNF_α production



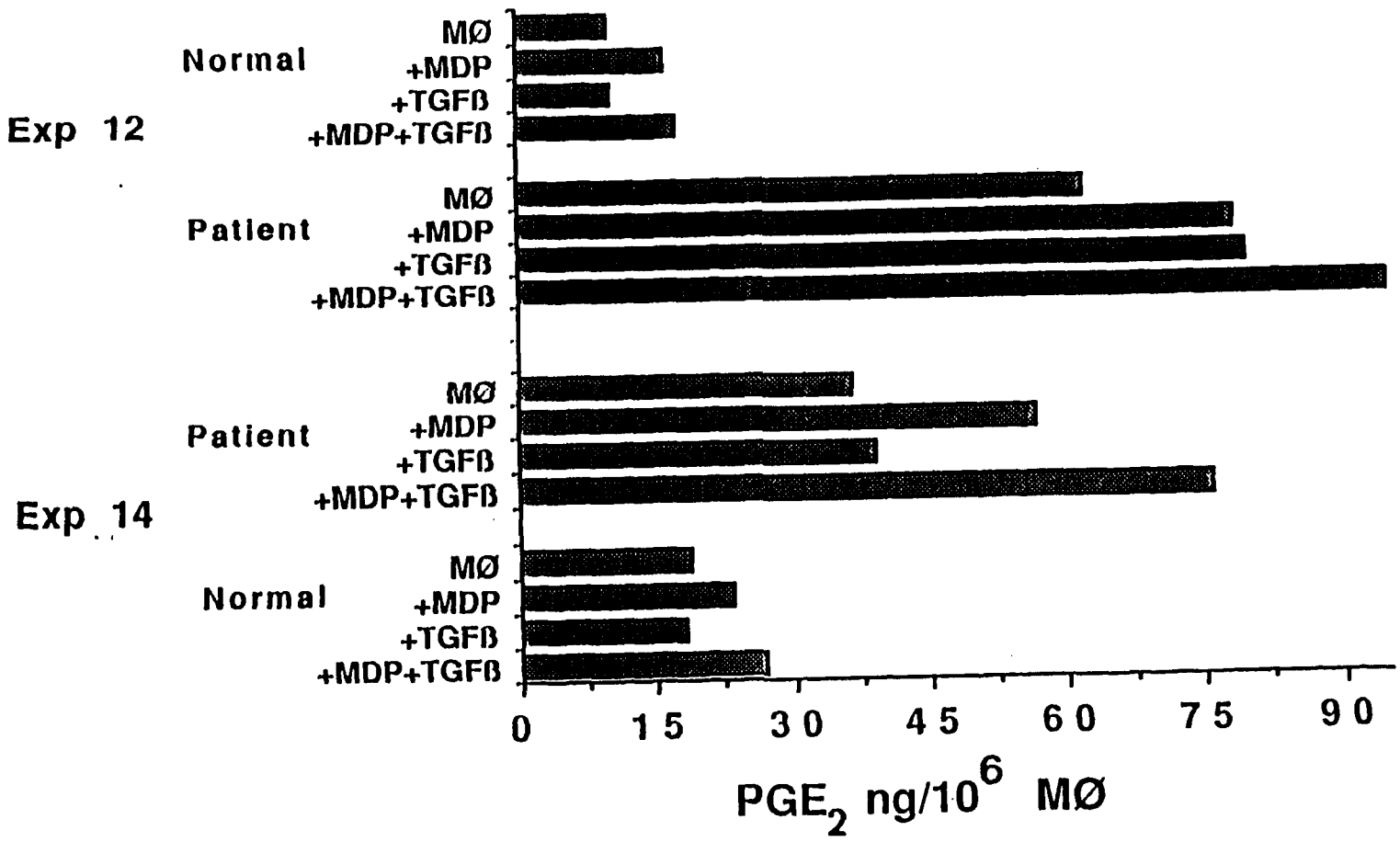
FcR1 crosslinked MØ further induced with IFN$_\gamma$ & MDP produce both cell-associated and secreted TNF$_\alpha$ while their TNFR are upregulated

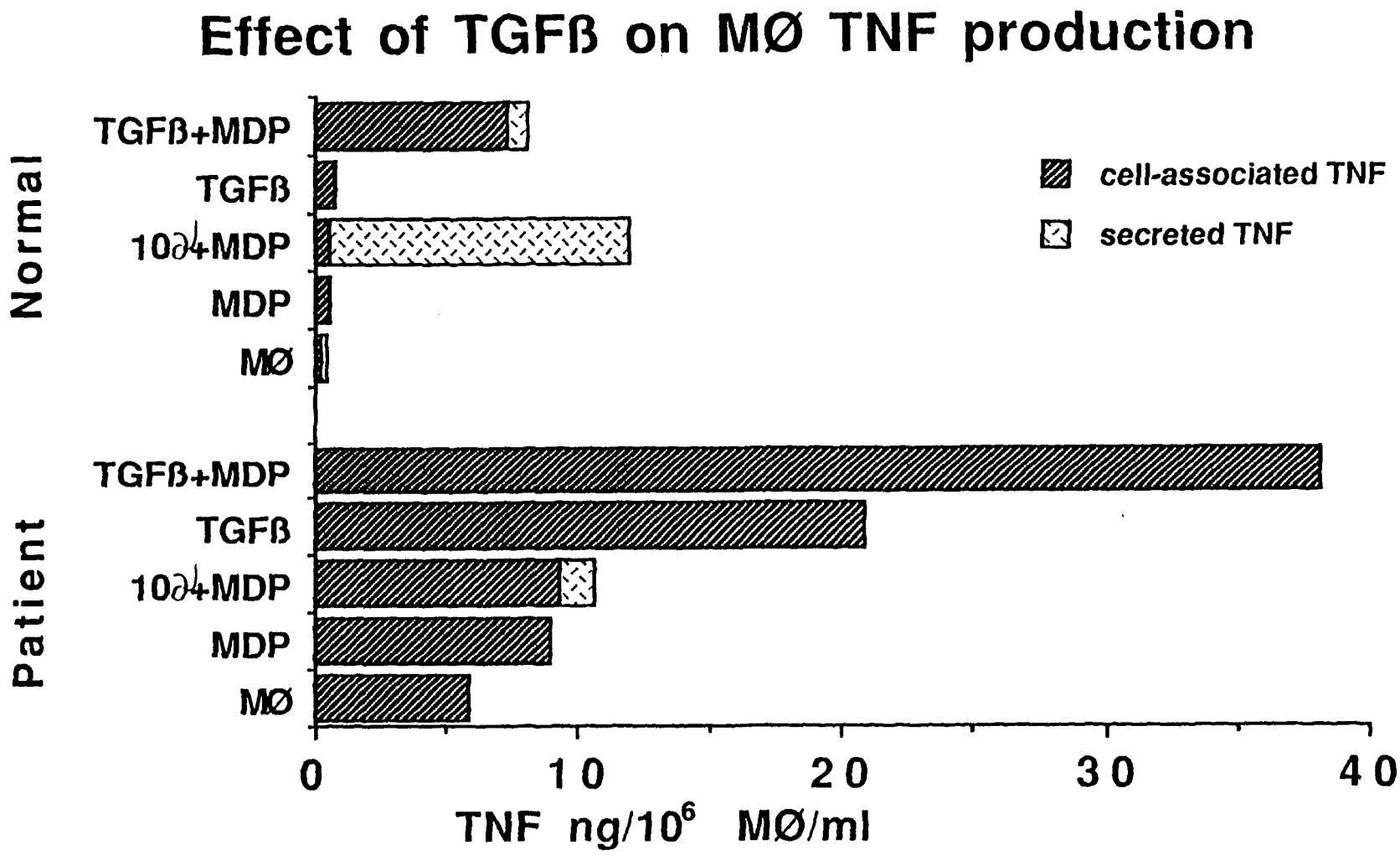


Increased TGFß Responses by Immunosuppressed Patients' MØ



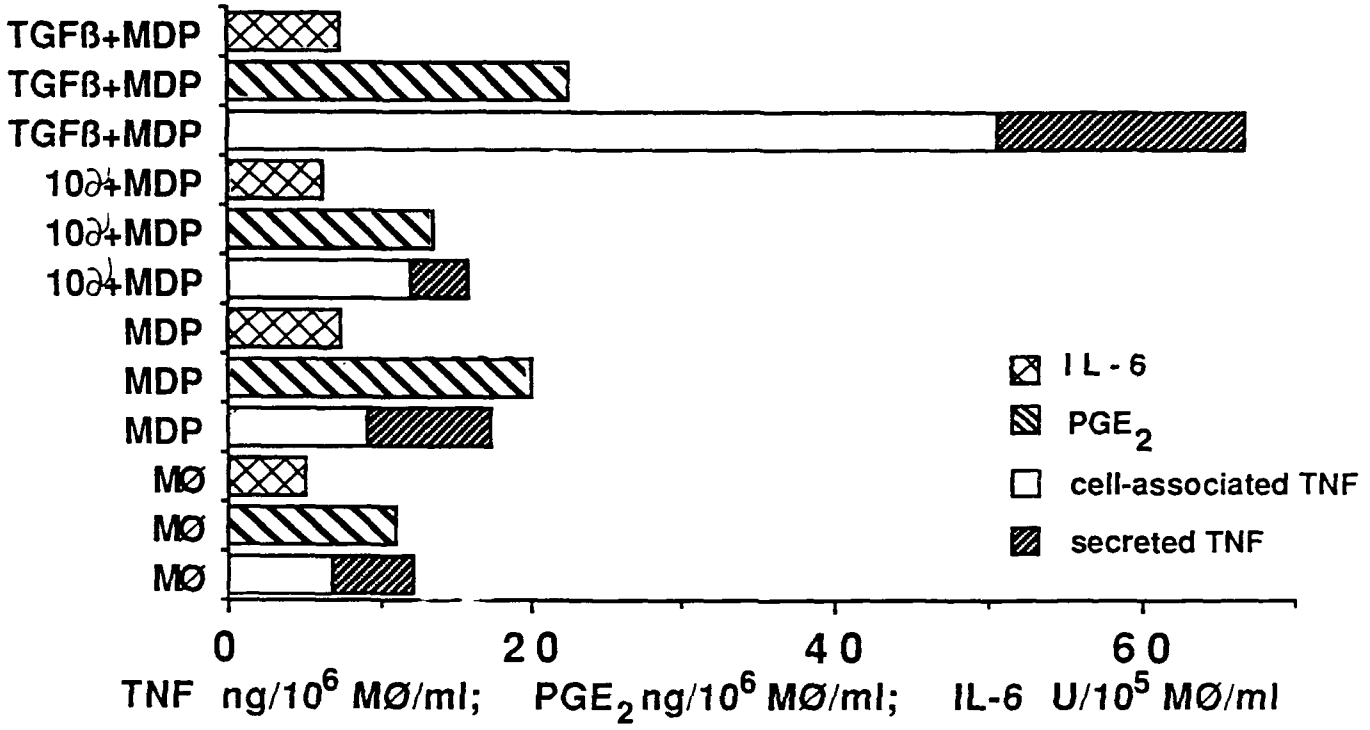
TGFß Increases Patients' MØ PGE Levels More Than Normals'




Effect of TGFß on MØ TNF production
Normal
Patient
TGFß+MDP
TGFß
10∂4+MDP
MDP
MØ
TGFß+MDP
TGFß
10∂4+MDP
MDP
MØ
cell-associated TNF
secreted TNF
0
10
20
30
40
TNF ng/10^6 MØ/ml

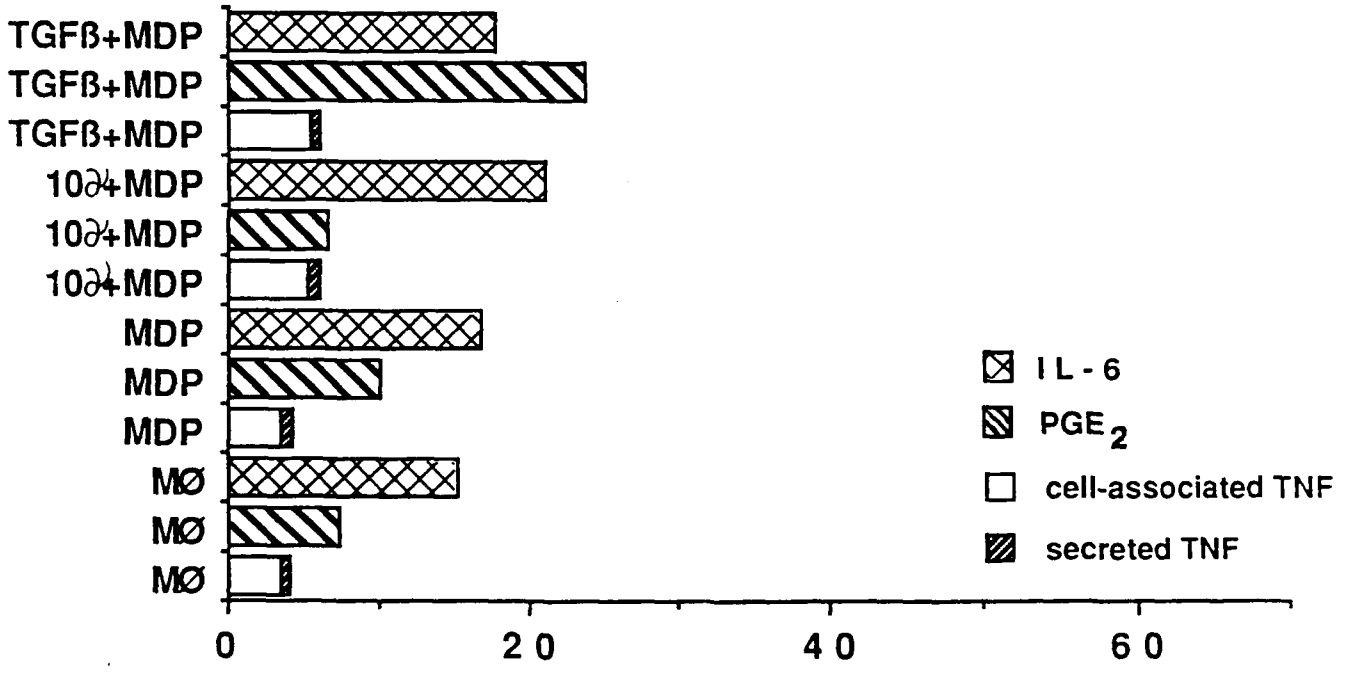
TGFß effect on monokine production

Patient

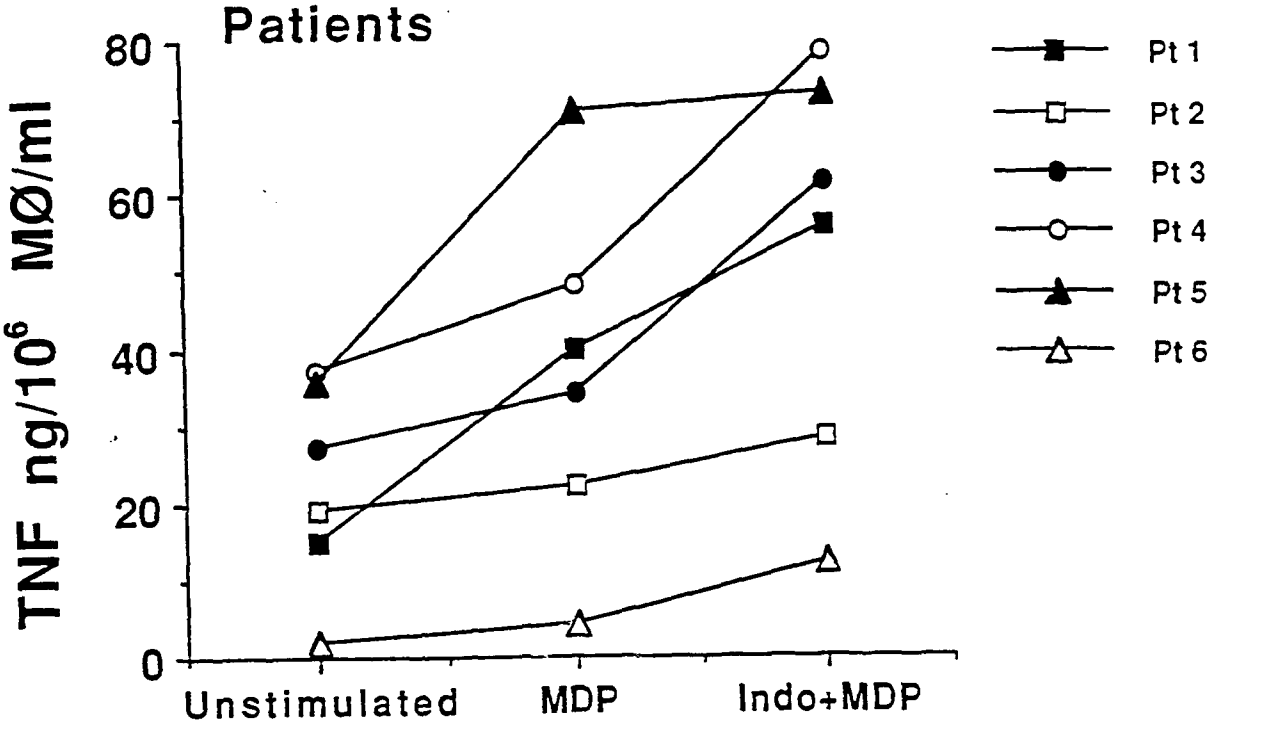


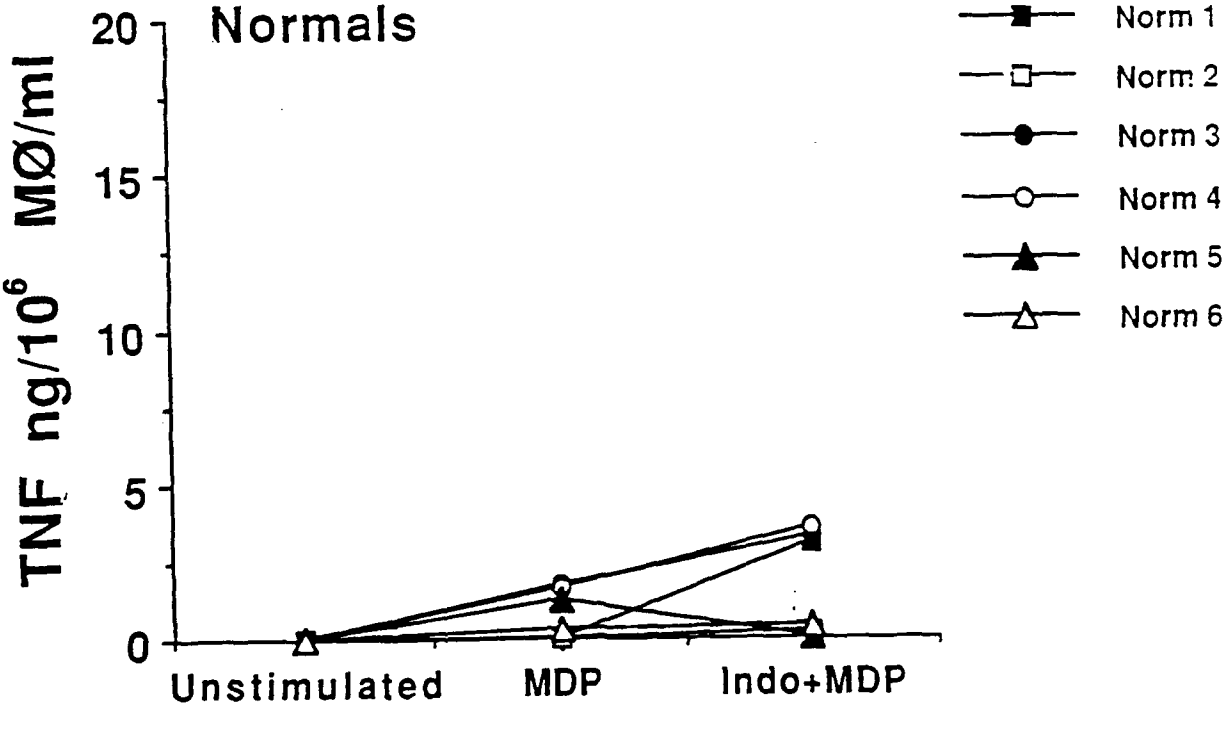
TNF ng/10^6 MØ/ml; PGE_2 ng/10^6 MØ/ml; IL-6 U/10^5 MØ/ml

Normal

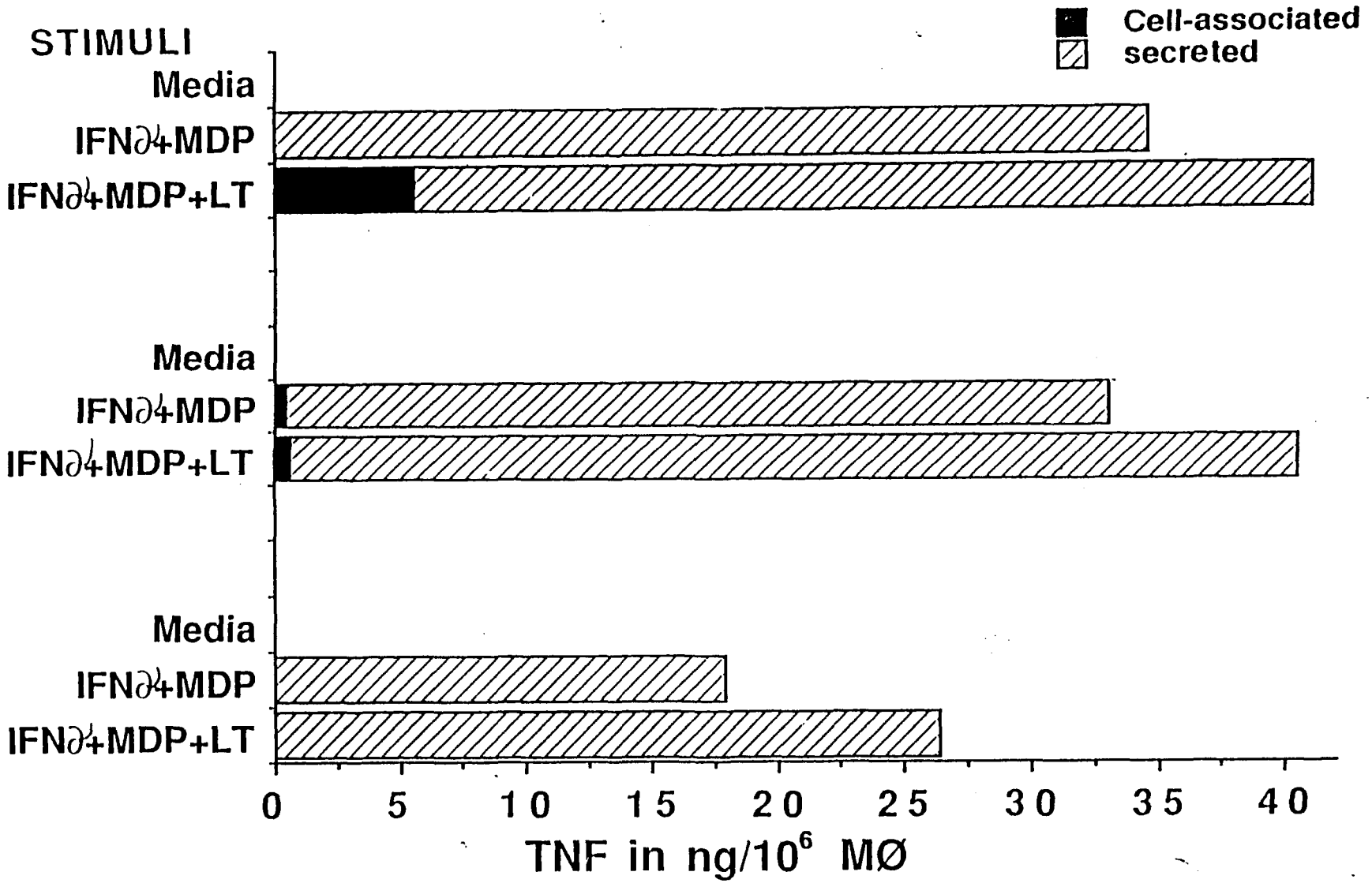


TNF ng/10^6 MØ/ml; PGE ng/10^6 MØ/ml; IL-6 U/10^5 MØ/ml

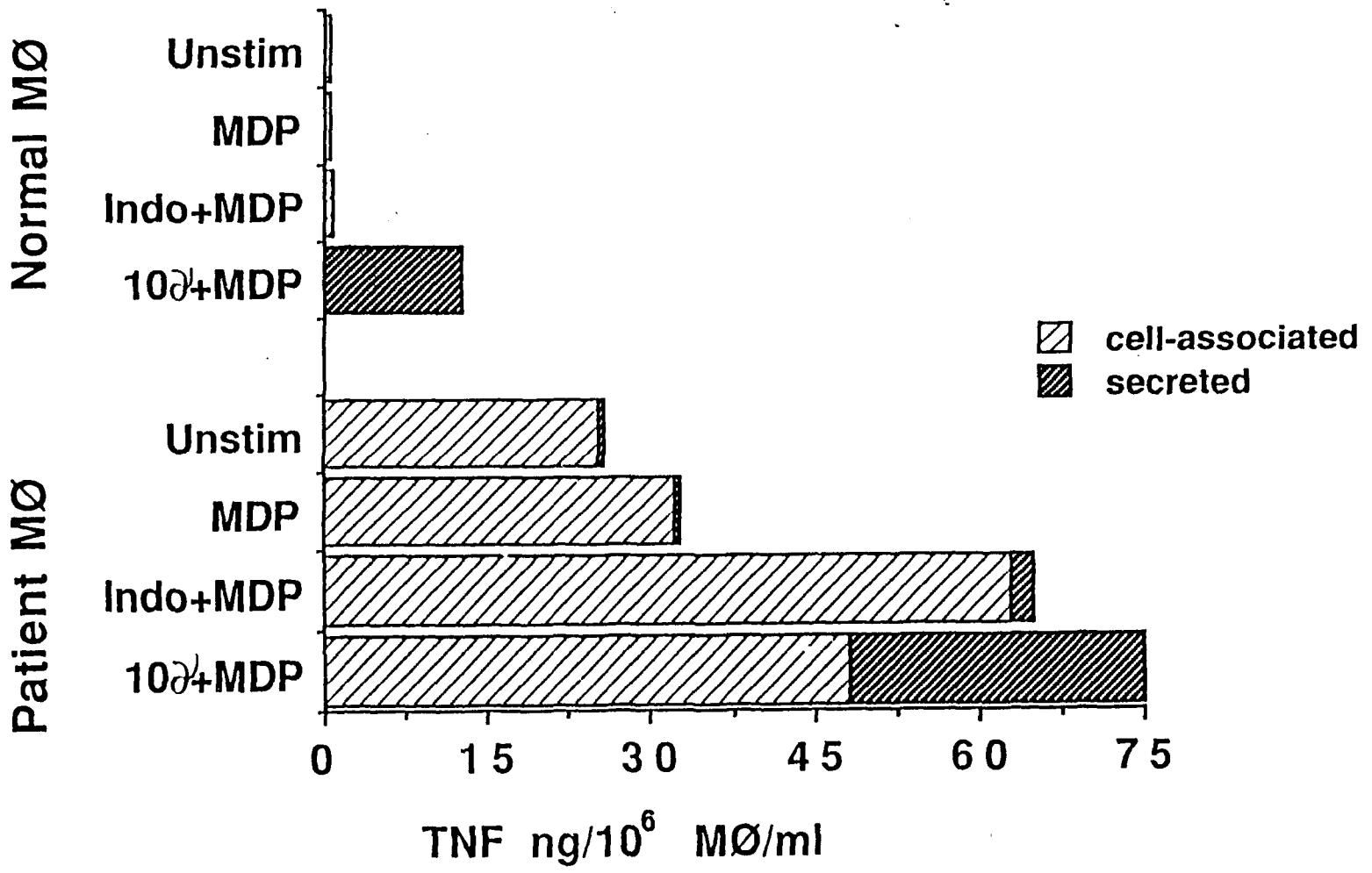

Patients
TNF ng/10^6 MØ/ml
80
60
40
20
0
Unstimulated
MDP
Indo+MDP
Pt 1
Pt 2
Pt 3
Pt 4
Pt 5
Pt 6


Normals
TNF ng/10^6 MØ/ml
20
15
10
5
0
Unstimulated
MDP
Indo+MDP
Norm 1
Norm 2
Norm 3
Norm 4
Norm 5
Norm 6

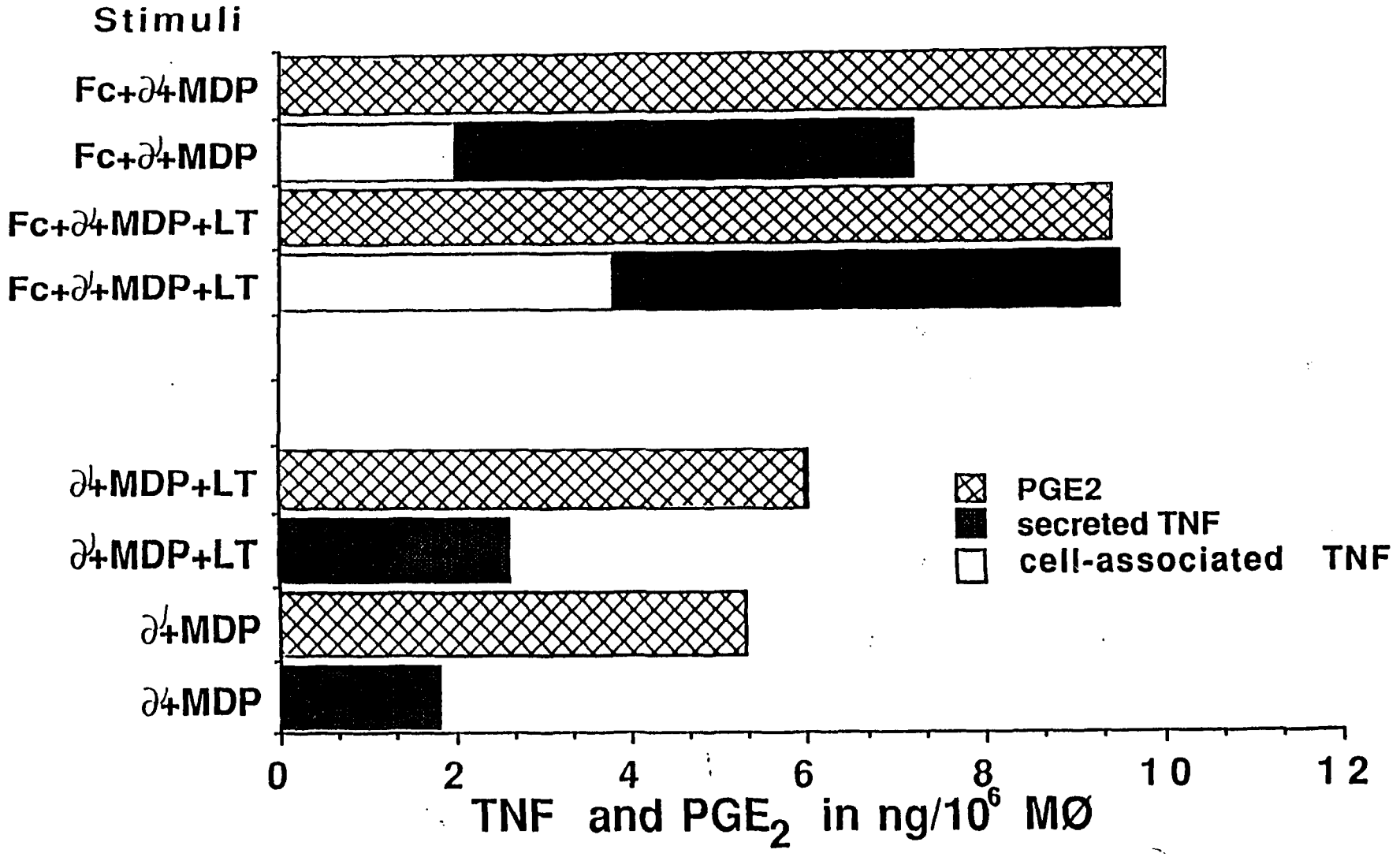
LTB_4 Increases IFN∂4+MDP Stimulated MØ TNF

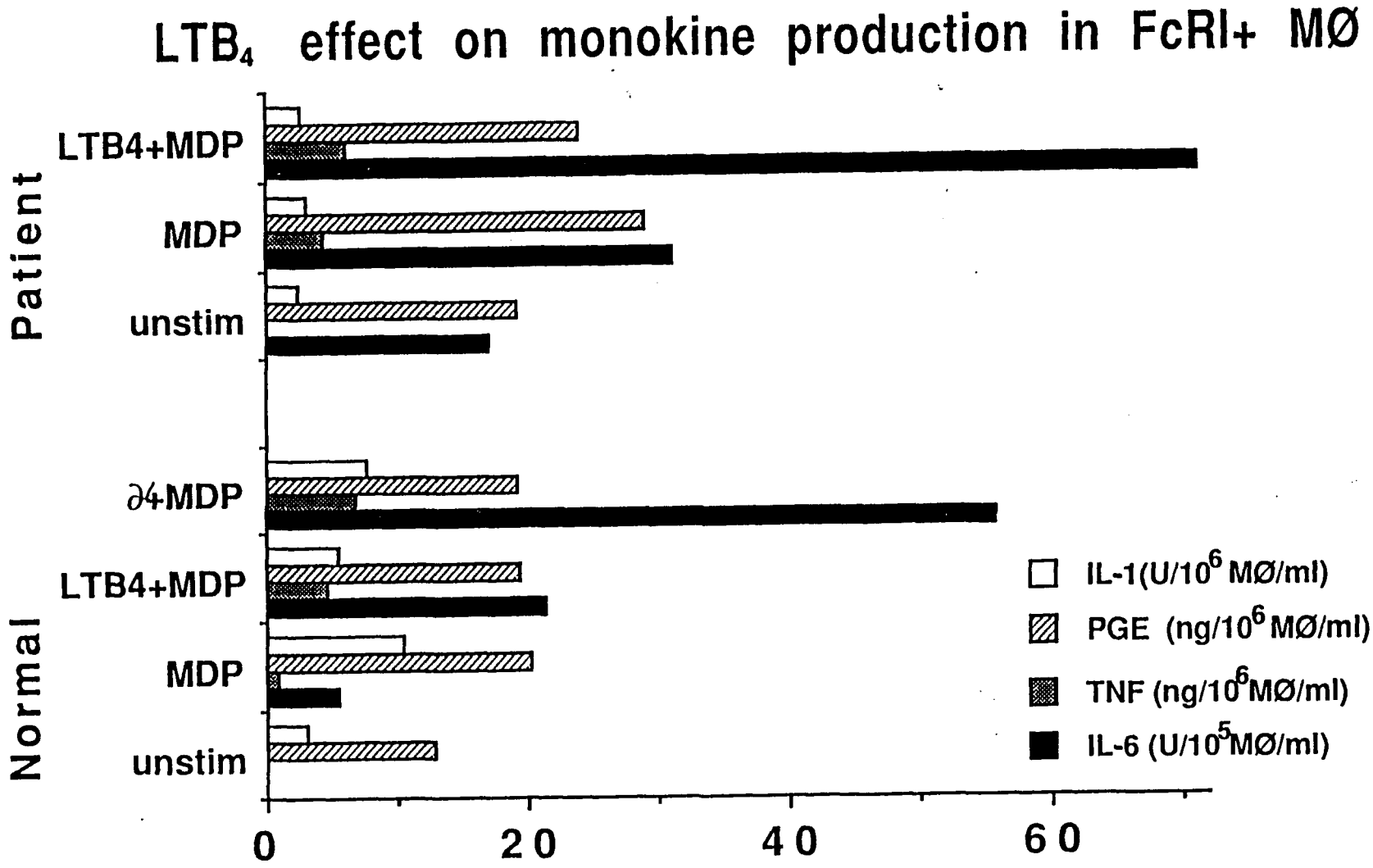


Indomethacin Increases Primarily Patient's Cell-associated TNF

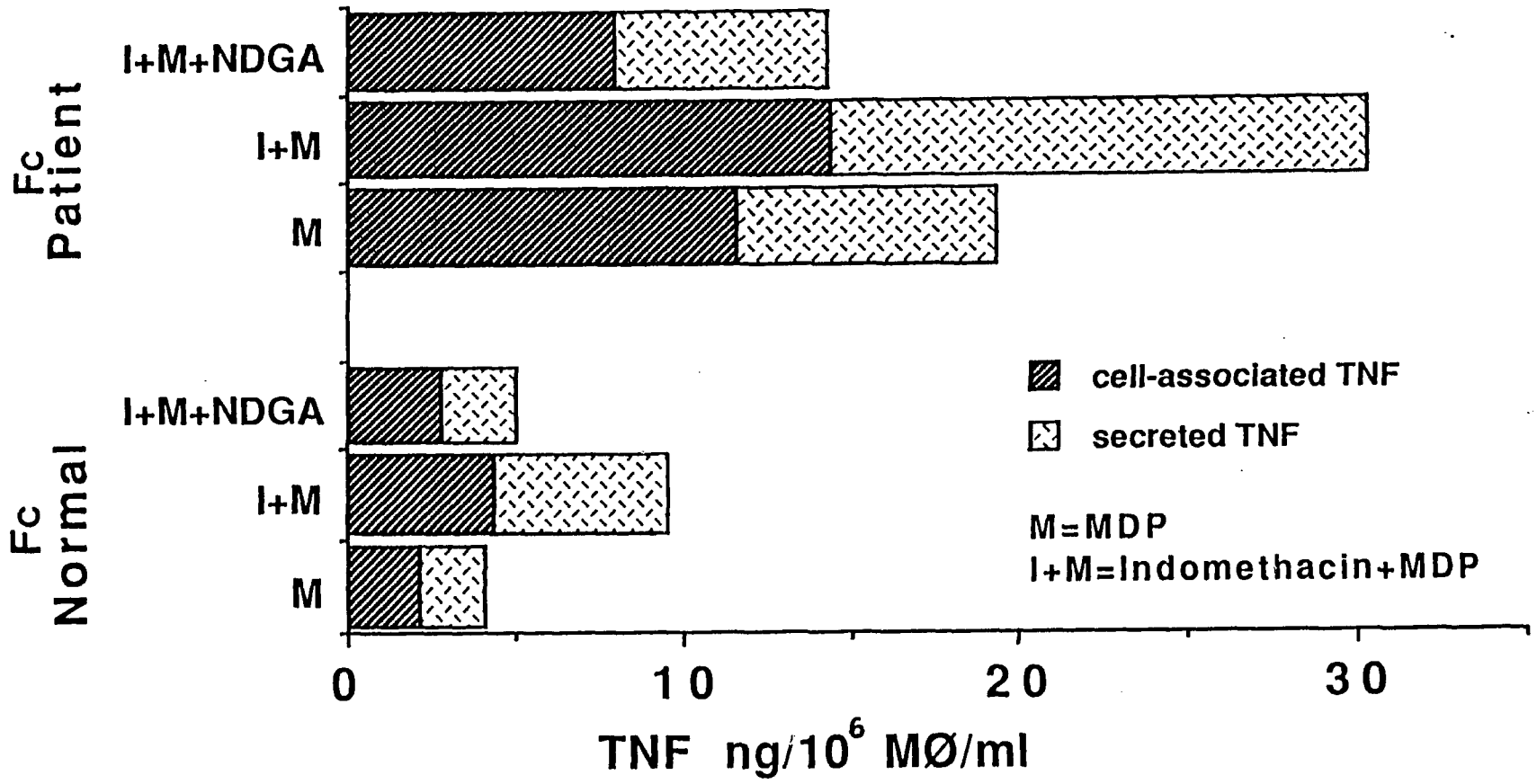


PGE_2 Levels are Unrelated to LTB_4 Effect on MØ TNF Levels

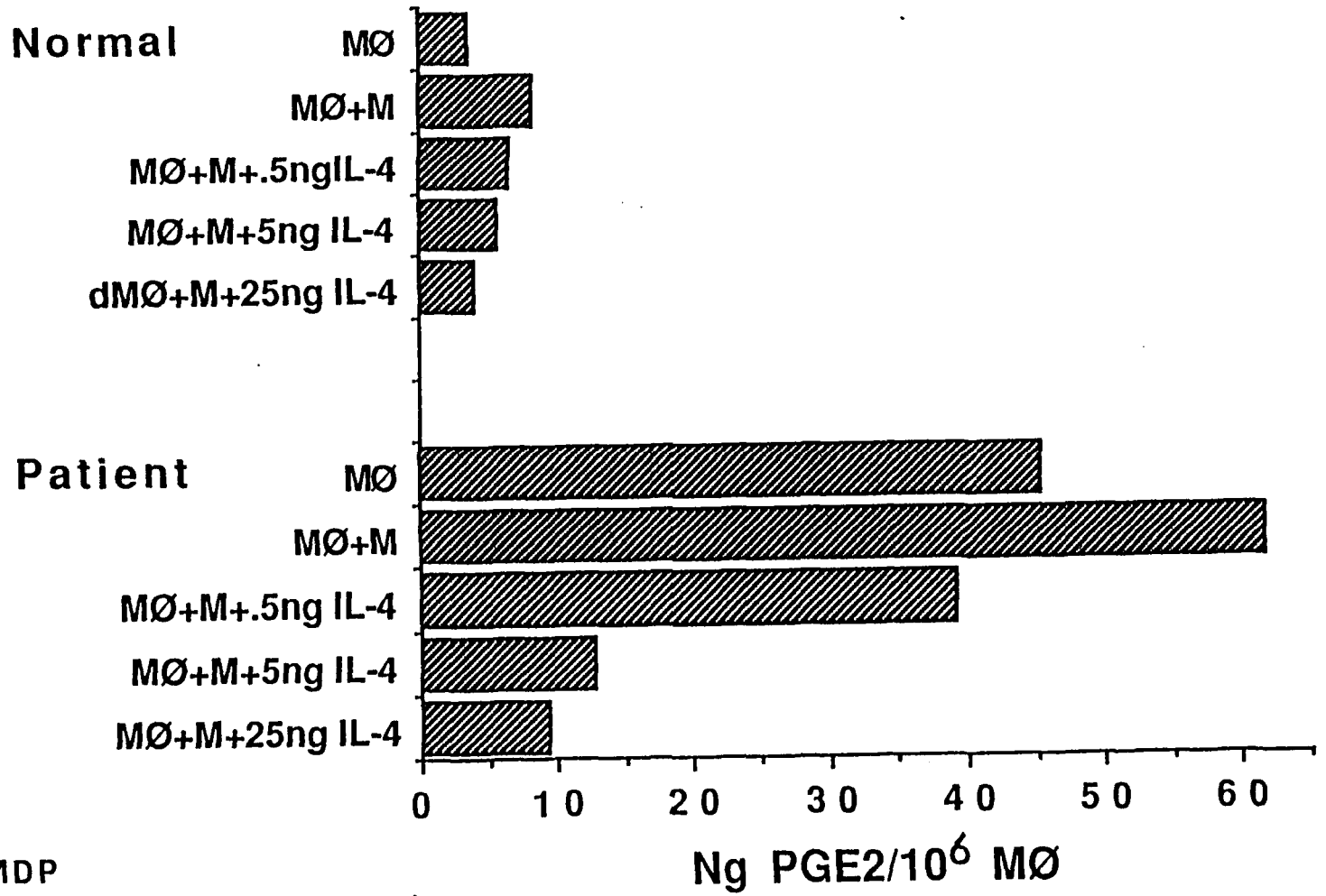



LTB$_4$ effect on monokine production in FcRI+ MØ
Patient
LTB4+MDP
MDP
unstim
Normal
∂4MDP
LTB4+MDP
MDP
unstim
0
20
40
60
IL-1(U/10^6 MØ/ml)
PGE (ng/10^6 MØ/ml)
TNF (ng/10^{6}MØ/ml)
IL-6 (U/10^{5}MØ/ml)

MØ TNF inhibition by lipoxygenase inhibitor

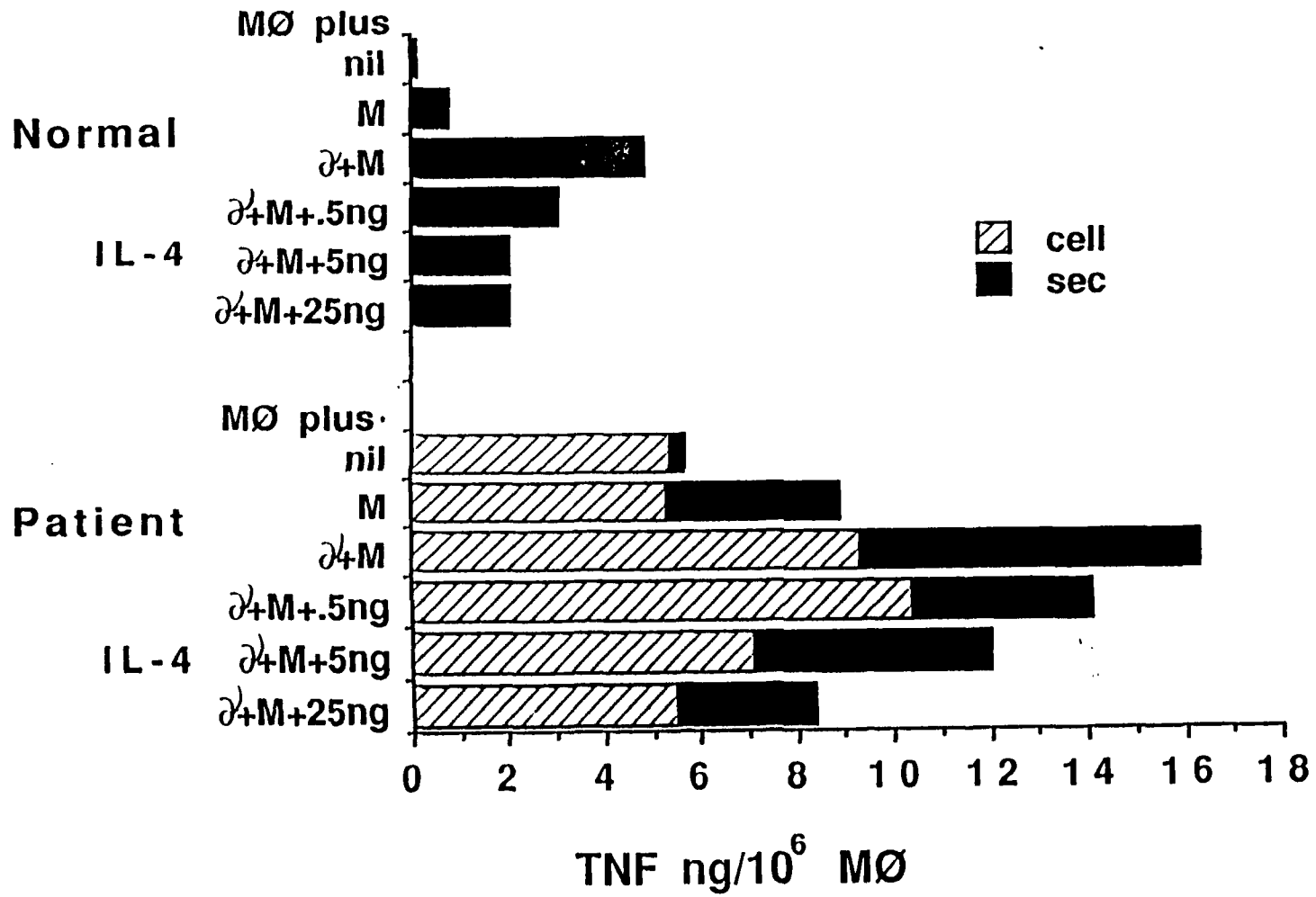


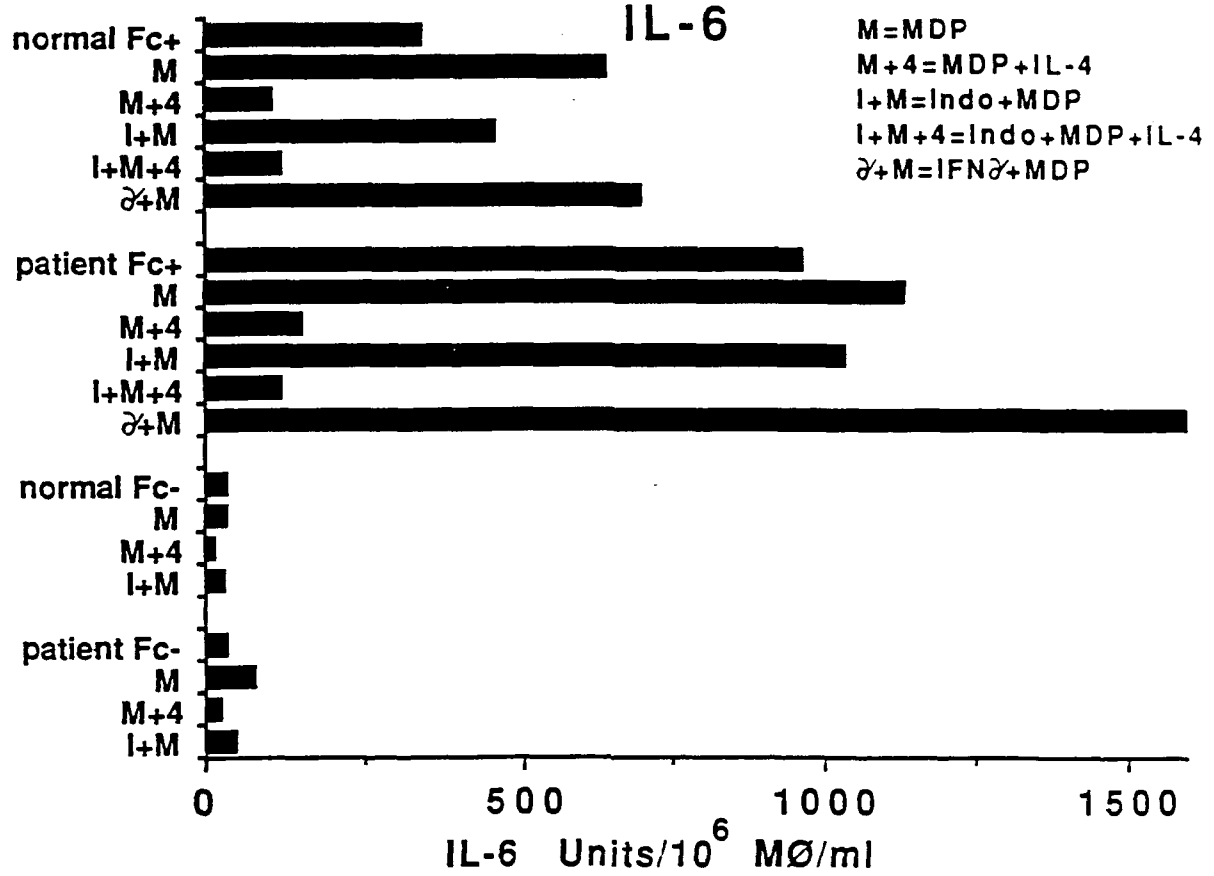
IL-4 downregulates Elevated MØ PGE2 Levels in Trauma Patients

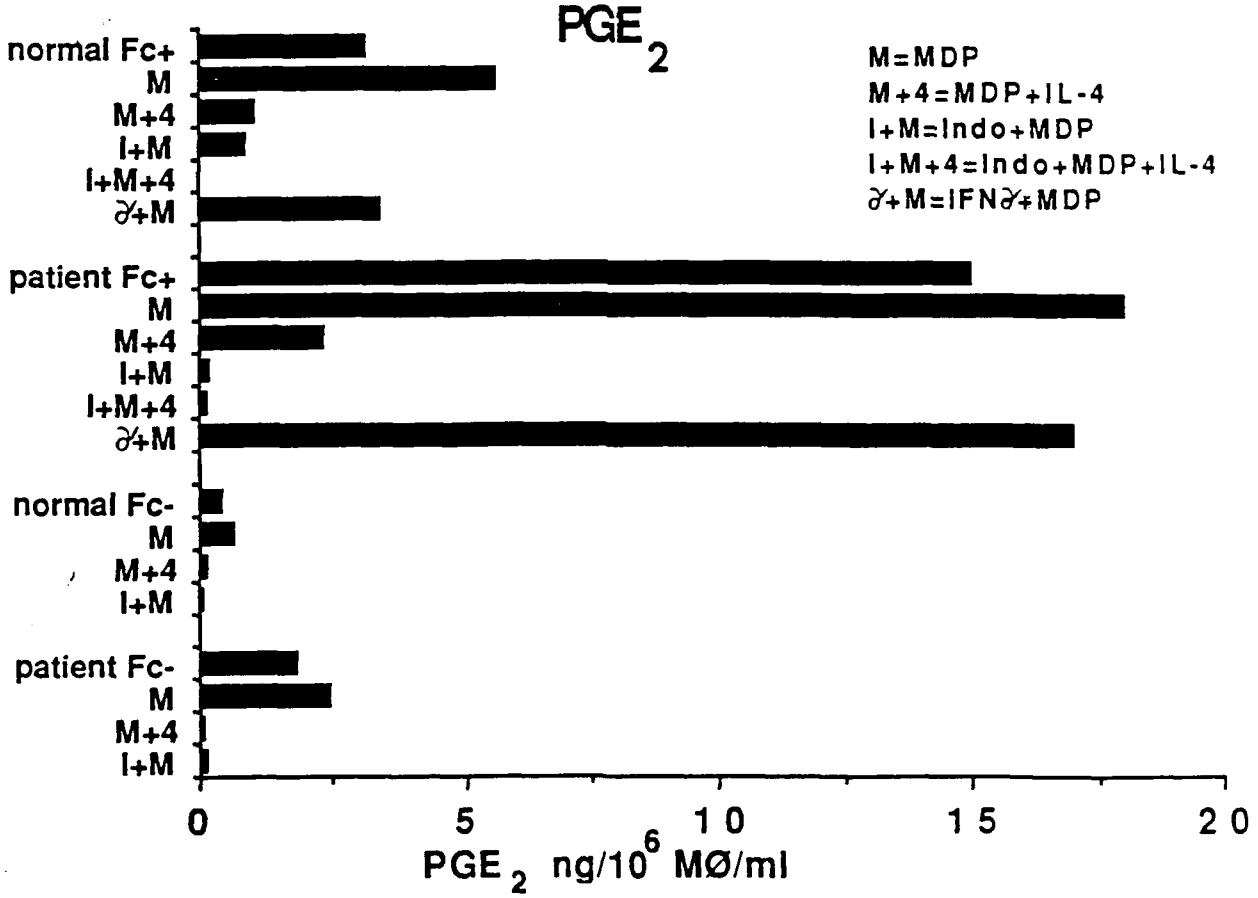


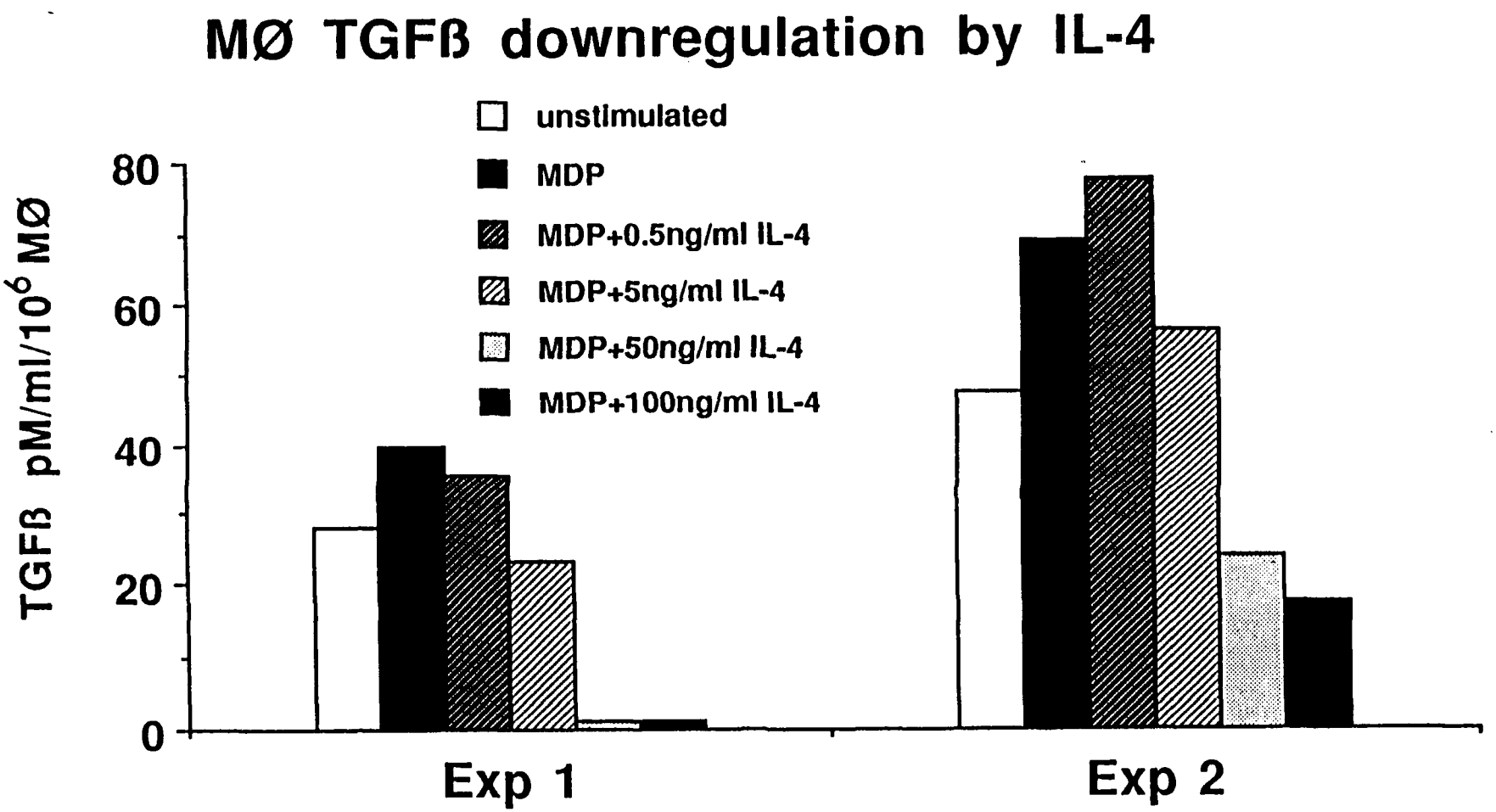
M=MDP

IL-4 Downregulates Elevated TNF Production by Trauma Patient's MØ

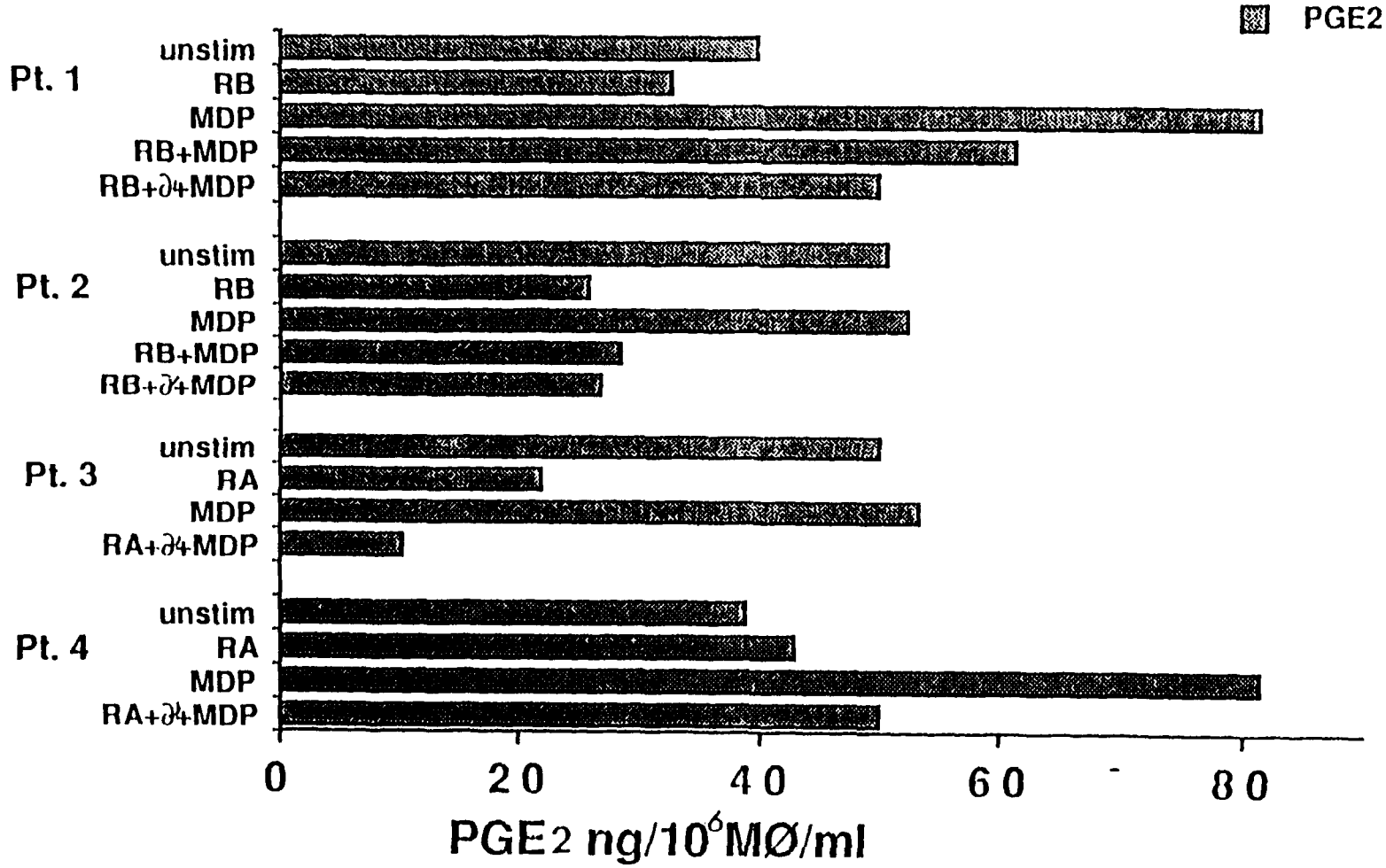



IL-6
M=MDP
M+4=MDP+IL-4
I+M=Indo+MDP
I+M+4=Indo+MDP+IL-4
∂+M=IFN∂+MDP
normal Fc+
M
M+4
I+M
I+M+4
∂+M
patient Fc+
M
M+4
I+M
I+M+4
∂+M
normal Fc-
M
M+4
I+M
patient Fc-
M
M+4
I+M
0
500
1000
1500
IL-6 Units/10^6 MØ/ml

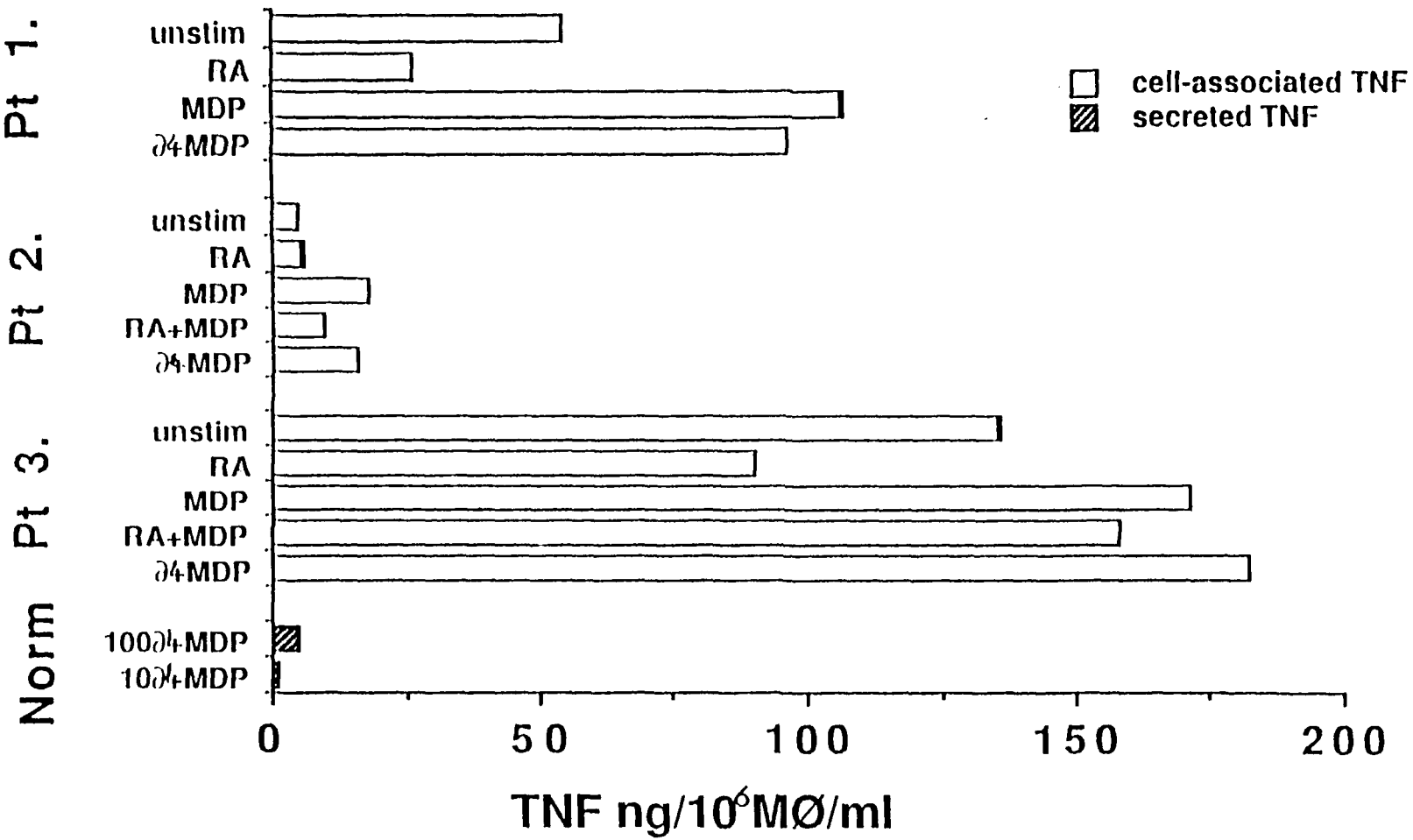

PGE2
M=MDP
M+4=MDP+IL-4
I+M=Indo+MDP
I+M+4=Indo+MDP+IL-4
∂+M=IFN∂+MDP
normal Fc+
M
M+4
I+M
I+M+4
∂+M
patient Fc+
M
M+4
I+M
I+M+4
∂+M
normal Fc-
M
M+4
I+M
patient Fc-
M
M+4
I+M
0
5
10
15
20
PGE2 ng/10^6 MØ/ml


MØ TGFß downregulation by IL-4
unstimulated
MDP
MDP+0.5ng/ml IL-4
MDP+5ng/ml IL-4
MDP+50ng/ml IL-4
MDP+100ng/ml IL-4
TGFß pM/ml/10^6 MØ
80
60
40
20
0
Exp 1
Exp 2

Downregulation of Trauma Patient MØ PGE2 by soluble PGG



DOWNREGULATION OF TRAUMA PATIENTS' MØ TNF LEVELS BY SOLUBLE PGG



Effect of Soluble PGGs on Trauma Patient MØ IL-6

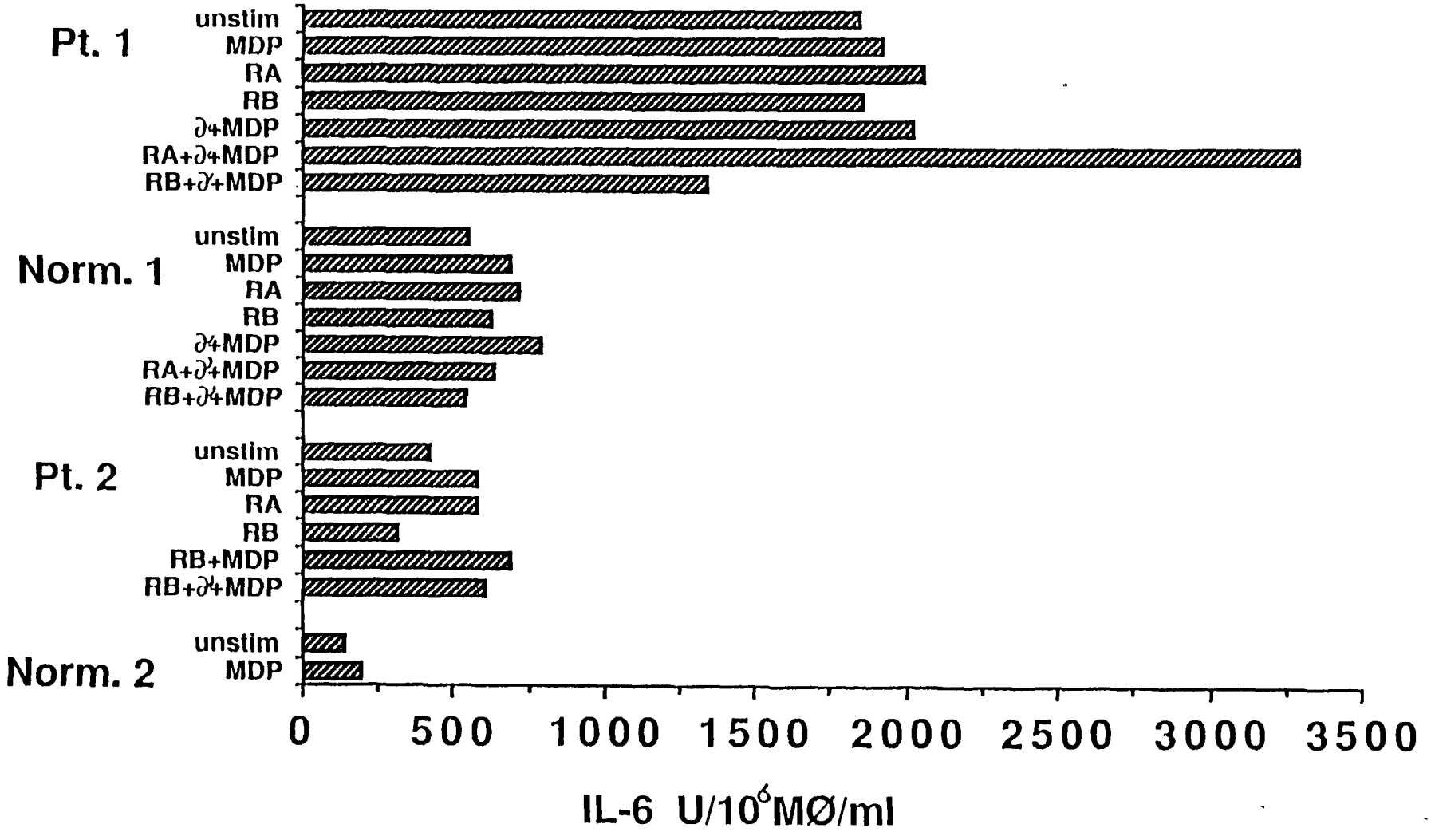


Figure Legends

Figure 1: T cell depleted MØ were separated into $FcRI^+$ (Fc^+) and $FcRI^-$ (Fc^-) MØ subpopulation by rosetting MØ with anti-Rh coated erythrocytes. The rosetting MØ represented the $FcRI^+$ and the non-rosetting MØ were the $FcRI^-$ MØ population. Fc^+/Fc^- MØ subpopulations were separated from immunocompromised patients 3,10 and 17 days post injury along with a healthy control in each experiment. The percentage of the patient's and normal's $FcRI^+$ MØ within the whole MØ population is shown. The average percentage of $FcRI^+$ MØ subpopulation obtained from 20 normal controls is also shown (normal).

Figure 2: Prostaglandin E_2 levels in controls, immunocompromised patients, and immunocompetent patients plotted for unseparated monocytes and the Fc^+ and Fc^- monocyte subsets. Fc^+ subset accounted for majority of elevation in prostaglandin E_2 levels.

Figure 3: Stimulation by crosslinking FcRI induces greater PGE_2 levels in patients' versus normals' MØ. Supernates from 16 hour cultures of equal numbers of $Fc_\gamma RI^+$ crosslinked (anti-Rh erythrocyte rosetted), non-crosslinked $Fc_\gamma RI^-$ (non-rosetting), remixed populations $Fc_\gamma RI^+/Fc_\gamma RI^-$ (MØ separated by anti-Rh erythrocyte rosetting and the combined at the proper ratio so that the whole population is also stimulated by FcRI crosslinking), and MØ (whole population before rosetting) were assessed for PGE_2 levels using the ELISA method. Results from four separate patients are shown and the normal control is the mean of the corresponding normal controls. Data is presented as nanograms per 10^6 recovered MØ per ml.

Figure 4: Elevated cell-associated TNF_α in 6 representative immunosuppressed trauma patients. TNF_α levels of MØ supernates (secreted) or cell lysates (cell-associated) as assayed in LM bioassay. All TNF_α activity is neutralizable with anti-TNF_α antibody. Median of normals assayed simultaneously with patients after suboptimal 10 U IFN_γ, and 20 μg/ml MDP stimulation.

Figure 5: Concomitantly increased MØ TNF_α and PGE_2 in immunosuppressed trauma patients. Data from 3 representative patients' monocytes with no in vitro stimulation or stimulated with suboptimal (10 U interferon γ + 20 μg MDP) were assayed for TNF_α (LM bioassay) and PGE_2 (ELISA) levels. TNF_α and PGE_2 values are nanograms/10^6 MØ/ml. Only normal MØ response to suboptimal stimulation is shown as median with range. Patient values (n=23) were statistically increased over normal, $p = <0.001$.

Figure 6: Trauma patients with increased proportions of $FcRI^+$ MØ have elevated MØ IL-6 levels. Patient and normal MØ were isolated, the $FcRI^+$ separated, and the ratio of $FcRI^+$ to $FcRI^-$ MØ determined. Medium normal ratios of $FcRI^+$ to $FcRI^-$ is 40/60. MØ were stimulated with 10 U IFN_γ +20 μg/ml MDP and their IL-6 production assessed in a hybridoma (B9) bioassay.

Figure 7: Patients with increased $FcRI^+$ MØ numbers have elevated levels of PGE_2, TNF_α and IL-6. Immunosuppressed trauma patients' and normals' MØ were segregated into $FcRI^+$ and $FcRI^-$ MØ subsets. The TNF_α, IL-6, and PGE_2

levels of these MØ populations were assayed as described and compared. MØ were stimulated with IFN_γ + MDP (top panel) or indomethacin (10^{-6} M) and MDP (bottom panel). Per cent of $FcRI^+$ or $FcRI^-$ in total MØ population shown at bottom.

Figure 8: $Fc_\gamma RI$ stimulation augments cell-associated MØ TNF_α. MØ were stimulated by Fc_γRI receptor crosslinking with anti-Rh erythrocyte rosetting. Fc^+ represents the rosetting population of MØ while Fc^- represents the non-rosetting MØ. MØ are the non-stimulated whole population. TNF_α was measured in MØ lysates (cell-associated) and supernates (secreted) using the LM bioassay. Data is presented as nanograms per 10^6 recovered MØ per ml. The ratios of the $Fc_\gamma RI^+$ and $Fc_\gamma RI^-$ MØ subsets (Fc^+/Fc^- MØ Ratio) within the unseparated MØ population (MØ) are shown for each experiment. There is a different individual blood donor for each experiment.

Figure 9: 10^5 peripheral blood mononuclear cells and adherence isolated and/or stimulated MØ were incubated with 24.5ng TNF_α phycoerythrin for 60 minutes. MØ were stimulated with 20μg/ml MDP or 100U/ml IFN_γ plus 20μg/ml MDP for 3 or 18 hours as indicated. Fluorescent measurements were performed on the Coulter EPICS profile with 530 SP (green) and 590 LP (red) emission filter. Percent TNFR positive cells was derived after adjusting the measurements window so that > 1% of control cells were positive. TNFR determinations are reported both as % TNFR positive cells and as mean TNFR fluorescent intensity of 10^4 measured cells. The production of TNF_α is also shown as measured in the stimulated MØ supernates (secreted TNF) and in the MØ lysates (cell-associated TNF) utilizing the L-M cell bioassay.

Figure 10: T cell depleted normals' peripheral blood MØ were further separated by crosslinking their $Fc_\gamma RI$ receptors through rosetting with anti-Rh coated erythrocytes. The rosetting, FcRI positive MØ were further investigated for $TNF_\alpha R$ expression and TNF_α production as described in Figure 9.

Figure 11: FcRI positive MØ subpopulation FcR1 was separated from peripheral blood MØ by crosslinking their FcRI receptors through rosetting with anti-Rh coated erythrocytes. FcRI crosslinked MØ were either only adherence induced (FcR1 + Ad) or additionally stimulated with 100μ IFN_γ plus 20μg/ml MDP (FcR1+Ad+γ+MDP) for 18 hours. MØ were labeled with TNF-phycoerythrin and percent of positive cells and mean fluorescence intensity was measured as described in Figure 9. Secreted TNF_α (in the MØ supernates) and cell-associated TNF_α (in the MØ lysates) activity (L-M assay) is shown on the stack columns.

Figure 12: Increased TGF_β responses in immunosuppressed patients' monocyte TGF_β responses from four representative immunosuppressed (left) and three immunocompetent patients (right) with their simultaneously run normal controls are shown. Each symbol represents the monocyte TGF_β levels of a different patient or individual. Monocyte TGF_β levels measured after stimulation with 20 μg/ml MDP and after 10 U/ml IFN_γ plus MDP are connected. The TGF_β levels of immunosuppressed patients' monocytes, solely MDP stimulated, exceeded the range of the maximally (IFN_γ plus MDP) stimulated monocyte TGF_β levels in immunocompetent patients or normals.

Figure 13: TGF_β increases patients' MØ PGE_2 levels more than normals. Equal numbers of both patients' and normals' MØ were cultured for 16 hours. MØ alone (media only), with MDP (20μg/ml), TGF_β (2.4ng/ml), or with MDP (20μg/ml) plus TGF_β (2.4ng/ml) were assessed for PGE_2 levels. PGE_2 was measured in the supernates by ELISA and presented as nanograms per 10^6 recovered MØ per ml.

Figure 14: Effect of TGF_β on MØ TNF_α production. Equal numbers of normals' or patients' MØ were cultured for 16 hours unstimulated (media only), with MDP (20μg/ml), IFN_γ (10U/ml) plus MDP (20μg/ml), TGF_β (2.4ng/ml), or with TGF_β (2.4ng/ml) plus MDP (20μg/ml). Secreted and cell-associated TNF_α was measured using the LM cell bioassay and reported as nanograms per 10^6 recovered MØ per ml.

Figure 15: TGF_β effect on monokine production. Both normals' and patients' MØ were cultured for 16 hours either alone (media only), with MDP (20μg/ml), IFN_γ (10U/ml) plus MDP (20μg/ml) or, with TGF_β (2.4ng/ml) plus MDP (20μg/ml). IL-6 was measured in the supernates using the B9 cell bioassay and reported as units per 10^5 recovered MØ per ml. The supernates were assayed for PGE_2 using an ELISA and reported as nanograms per 10^6 recovered MØ per ml. Both cell-associated and secreted TNF_α were measured using the LM cell bioassay and presented as nanograms per 10^6 recovered MØ per ml.

Figure 16: Indomethacin augments TNF_α levels in patients' activated MØ. Equal numbers of MØ were cultured either unstimulated (media only), MDP (20μg/ml) stimulated, or indomethacin (10^{-6}) plus MDP (20μg/ml) stimulated. After 16 hours of culture, the TNF_α levels were measured using the LM bioassay. The data is reported as total TNF_α in nanograms per 10^6 recovered MØ per ml.

Figure 17: LTB_4 increases IFN_γ + MDP stimulated MØ TNF_α. Equal numbers of MØ were cultured for 16 hours in media only, IFN_γ (100U/ml) plus MDP (20μg/ml) or, IFN_γ (100U/ml) plus LTB_4 (10^{-7}M) plus MDP (20μg/ml). Both secreted and cell-associated TNF_α was measured (as in Fig. 8) using the LM bioassay. Data is presented as nanograms per 10^6 recovered MØ per ml.

Figure 18: Indomethacin primarily increases patients' cell-associated TNF_α. Equal numbers of either patients' or normals' MØ were cultured for 16 hours without stimuli (media only), with MDP (20μg/ml), with indomethacin (10^{-6}M) plus MDP (20μg/ml), or with IFN_γ (10U/ml) plus MDP (20μg/ml). Cell-associated and secreted TNF_α was measure using the LM bioassay. Data is presented as nanograms per 10^6 recovered MØ per ml.

Figure 19: LTB_4 stimulates increased cell-associated and secreted TNF_α levels in $Fc_\gamma RI$ crosslinked normal MØ independent of PGE_2 levels. Equal numbers of MØ either FcRI crosslinked (anti-Rh erythrocyte rosetting) or whole MØ population (non-rosseted) were cultured with IFN_γ (100U/ml) plus MDP (20μg/ml) or IFN_γ (100U/ml) plus MDP (20μg/ml) plus LTB_4 (10^{-7}M). MØ supernates were assayed for PGE_2 using the ELISA Method and for secreted TNF_α in the LM bioassay. Cell lysates were also assayed for cell-associated TNF_α. Both TNF_α and PGE_2 were reported in nanograms per 10^6 recovered MØ per ml.

Figure 20: LTB_4 effect on monokine production in $Fc_\gamma RI^+$ MØ. $Fc_\gamma RI^+$ MØ were selected by rosetting with anti-Rh coated erythrocytes. Equal numbers of $Fc_\gamma RI^+$ MØ from either patients' or normals' were cultured for 16 hours unstimulated (media only), with MDP (20μg/ml) or, with LTB_4 (10^{-7}M) plus MDP (20μg/ml). IL-1 was measured in the MØ supernates using the D10.G4.1 cell bioassay and reported as Units per 10^6 recovered MØ per ml. PGE_2 was assayed in the supernates by ELISA and reported as nanograms per 10^6 recovered MØ per ml. TNF_α was measured using the LM bioassay and presented as nanograms per 10^6 recovered MØ per ml. The MØ supernates were also assayed for IL-6 using the B9 cell bioassay and reported as Units per 10^5 recovered MØ per ml.

Figure 21: Lipoxygenase inhibitor decreased indomethacin enhanced TNF_α levels. Both normal and patient MØ were FcRI crosslinked (anti-Rh erythrocyte rosetted) and cultured with either MDP alone (20μg/ml), indomethacin (10^{-6}M) plus MDP (20μg/ml), or indomethacin (10^{-6}M) plus MDP (20μg/ml) plus the lipoxygenase inhibitor Nordihydroguaiaretic acid (NDGA) (40μM). After 16 hours of culture, both secreted and cell-associated TNF_α were measured using the LM cell bioassay. TNF_α is presented as nonograms per 10^6 recovered MØ per ml.

Figure 22: IL-4 downregulates elevated MØ PGE_2 levels in trauma patients. Equal numbers of both patients' and normals' MØ were cultured either without stimuli (media only), with MDP (20μg/ml), or with IL-4 (at concentrations of 0.5ng/ml, 5ng/ml and 25ng/ml) in combination with MDP (20μg/ml). PGE_2 was assayed using an ELISA and reported as nanograms per 10^6 recovered MØ per ml.

Figure 23: IL-4 downregulates elevated TNF_α production by trauma patients' MØ. Equal numbers of normals' and patients' MØ were cultured for 16 hours with either media only, MDP (20μg/ml), IFN_γ (10U/ml) plus MDP (20μg/ml), or IFN_γ (10U/ml) plus MDP (20μg/ml) plus IL-4 (.5ng/ml, 5ng/ml or 25ng/ml). Both secreted and cell-associated TNF_α was measured using the LM cell bioassay. Data is presented as nanograms per 10^6 per ml.

Figure 24: Concomitant downregulation of MØ IL-6 and PGE_2 production by IL-4 in trauma patients. MØ from trauma patients and normal controls were stimulated via $Fc_\gamma RI$ crosslinking. $Fc_\gamma RI$ stimulated (Fc^+) and $Fc_\gamma RI$ unstimulated (Fc^-) MØ population were further stimulated with a combination of 20μg/ml MDP (M), 5ng/ml IL-4 (4), 10^{-6}M Indomethacin (I), 100U/ml IFN_γ (γ) as indicated. MDP stimulation was always added 4 hours after the IFN_γ, Indo or IL-4 priming. MØ supernates were harvested after a total of 16 hours stimulation to determine the IL-6 (B9 assay) and PGE_2 (ELISA) activity.

Figure 25: Adherence isolated MØ were cultured at $1x10^6$/ml unstimulated, with MDP (20μg/ml), or in the absence or presence of increasing doses of IL-4 (MDP + 0.5ng/ml IL-4, etc.) for 20 hours. IL-4 was added four hours before MØ stimulation with MDP. MØ TGF_β activity was determined in the acid-tested MØ supernates in the Mv1Lu bioassay.

Figure 26: Trauma patients' MØ (Pt.1, Pt.2) were cultured at $1x10^6$/ml in medium (unstim), stimulated with soluble glucan RB (20μg/ml RB), 20μg/ml MDP or with a combination of 20μg/ml RB plus 20μg/ml MDP (RB + MDP) or 20μg/ml

RB plus 100U/ml IFN$_\gamma$ plus 20μg/ml MDP (RB + γ + MDP). In case of Pt. 3 and Pt. 4, soluble glucan RA was used at 20μg/ml concentration. MØ supernates after 20 hours stimulation were collected and tested for PGE_2 activity in an ELISA assay.

Figure 27: MØ from trauma patients were cultured in medium (unstim), stimulated with 20μg/ml soluble glucan RA, 20μg/ml MDP (MDP) or with a combination of 10U/ml/10^6 MØ IFN$_\gamma$ plus 20μg/ml MDP (γ +MDP). MØ from normal controls were maximally induced with 100U/ml IFN$_\gamma$ plus 20μg/ml MDP (100γ + MDP) or subsequently stimulated with 10U IFN$_\gamma$ plus 20μg/ml MDP (10$_\gamma$ + MDP). Both patients' and normals' MØ were stimulated for 20 hours. Secreted MØ TNF$_\gamma$ in the MØ supernates and cell-associated MØ TNF$_\gamma$ activity in the MØ-lysates were determined in the L-M cell bioassay.

Figure 28: Trauma patients' and normals' MØ were adherence isolated (unstim) and stimulated with 20μg/ml MDP (MDP), 20μg/ml soluble glucan RA, 20μg/ml soluble glucan RB (RB) or with a combination of 100U/ml IFN$_\gamma$ plus 20μg/ml MDP in the absence (γ + MDP) or presence of RA or RB (RA + γ + MDP; RB + γ + MDP). MØ supernates were colleced after 20 hours stimulation and tested for IL-6 activity in the B9 hybridoma assay.

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Glossary of Abbreviations:

AA861	Inhibitor of both cyclo-oxygenase and lipoxygenase
Ab	Antibody
Ag	Antigen
DCF	Dichlorofluorescein
DCFH-DA	Dichlorofluorescein diacetate
DR	Human immune response antigen
DQ	Primary Ia antigen on human monocyte
DTH	Delayed type hypersensitivity
EA	Erythrocyte-antibody complex
FBS	Fetal bovine serum
FcRI	72kd receptor for IgG
Fura-2	The free acid of a fluorescent molecule which strongly binds Ca^{++} and then alters its fluorescent emission wavelength.
Fura-2AM	Fura-2 bound to acetoxymethyl ester so it becomes permeable to the cell and is readily taken up.
^{3}H-TDR	Tritiated Thymidine
HLA	Human lymphocyte antigen
Ia	Immune response antigen
IgG	Immunoglobulin gamma
IFN_{γ}	Interferon gamma
IL	Interleukin
IL-1	Interleukin 1-a monokine involved in T cell proliferation
$IL\text{-}1_{\alpha}$	Interleukin 1 alpha
$IL\text{-}1_{\beta}$	Interleukin 1 beta
IL-1 inh	IL-1 inhibitor
IL-1R	Interleukin 1 receptor
IL-2	Interleukin 2-a lymphokine involved in T cell proliferation
IL-4	Interleukin 4 - B cell stimulatory factor; mast cell activating factor
IL-6	Interleukin 6 - hybridoma/plasmocytoma growth factor 1; B cell stimulatory factor 2, hepatocyte stimulatory factor
IL-8/NAF	Neutrophil activating factor; MIP-2 family
Indo	Indomethacin
LP	Leukocyte pyrogen
LTB_4	Leukotriene B_4
MDP	Muramyl dipeptide - synthetic bacterial cell wall analogue
MIP-1	Monocyte inflammatory protein 1
MIP-2	Monocyte inflammatory protein 2
MØ	Monocyte, macrophage
mRNA	Messenger ribonucleic acid
Mv1Lu	Mink Lung
NDGA	Nordihydroguaiaretic acid
OKM5	Monoclonal antibody specific for monocytes
PAF	Platelet activating factor
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
PMN	Polymorphonucleocytes
PMNL	Polymorphonuclear leukocytes
rIL-1	recombinant Interleukin-1
TGF_{β}	Transforming Growth Factor beta
TNF_{α}	Tumor Necrosis Factor alpha
TNFR	Tumor Necrosis Factor Receptor